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Understanding the role of B cells during Leishmania amazonensis infection

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by

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Chapter 1

GENERAL INTRODUCTION

Leishmaniasis

Introduction and epidemiology

Leishmaniasis is a vector-borne zoonotic disease caused by obligate intracellular protozoan parasites of the genus *Leishmania*. This disease is classified geographically into New World, which occurs in Central and South America and Old World, present in Africa, Asia, the Middle East and the Mediterranean (19, 65). There are an estimated 12 million cases of leishmaniasis worldwide with 2 million new cases per year (65). Although endemic in the tropics, sporadic cases have been reported in the United States, usually from travelers returning from an endemic area. The incidence of leishmaniasis in the United States is of growing concern however, as greater than 600 cases of cutaneous leishmaniasis and 4 cases of visceral leishmaniasis were diagnosed in the United States in 2004, primarily due to an increasing number of soldiers returning from Iraq, Kuwait, and Afghanistan (108). In 2000, *Leishmania infantum*, the causative agent of zoonotic visceral leishmaniasis, was found to have killed four foxhounds in New York (27). There is a possibility for spread of this disease to both dogs and humans, as dogs are one of the primary domestic reservoirs in locations endemic for human disease (22). Because *Leishmania* infection has been shown to exhibit primary and secondary resistance to standard treatments (92, 93), continued research is vital to determine protective immune responses to this parasite and to formulate new vaccination and treatment options.

Leishmaniasis is transmitted by the bite of an infected female sand fly, which tend to feed during dusk (19). Old World disease is primarily transmitted by the *Phlebotomus* species of sand fly, while New World disease is transmitted by the *Lutzomyia* species. *Leishmania* parasites have two distinct morphologies; the infective form is the elongated, flagellated promastigote, which is injected into the mammalian host by the sand fly. Promastigotes enter host cells, specifically macrophages and dendritic cells, via receptor-mediated phagocytosis thus establishing infection within intracellular parasitophorous vacuoles. Once inside cells the parasite then becomes a rounded, non-flagellated amastigote which survives and multiplies within parasitophorous vacuoles and eventually leads to cell lysis and local spread of parasites. Some forms of *Leishmania* can also become disseminated via lymphatic and/or hematogenous spread, thus causing systemic infection. The lifecycle is completed when the sand fly takes a blood meal from an infected host, ingesting host cells containing amastigotes. Amastigotes travel to the midgut of the insect where they become promastigotes and multiply (Figure 1).

Pathophysiology of Leishmaniasis

Leishmaniasis is a disease that affects both humans and other mammalian species; dogs and rodents being the primary reservoirs for human disease in endemic regions (21). There are 21 classified species of *Leishmania* that can cause disease. Infection with the parasite can lead to four main categories of disease; cutaneous leishmaniasis, diffuse cutaneous leishmaniasis, mucocutaneous leishmaniasis and visceral leishmaniasis (19, 65). Cutaneous leishmaniasis (CL) is

the most common form of the disease and in the Old World is commonly caused by *L. major* and *L. tropica* (19, 65). In the New World the primary species that cause CL include *L. mexicana*, *L. braziliensis* and *L. panamensis* (19). There are 1.5 million new cases of CL annually worldwide (65). CL manifests as focal to multifocal skin lesions that can occur at the site of the infected sand fly bite (105). Lesions develop 1-12 weeks after infection and typically consist of one or more painless cutaneous ulcers at the site of inoculation characterized by a necrotic center with raised margins covered by a crusty exudate. These lesions may heal, but lymphangitis and secondary bacterial infections also occur (40). CL caused by *Leishmania (L.) major* infection presents as localized cutaneous lesions and disease distribution includes the Middle East, Africa, and central Asia. Infection with *L. amazonensis*, another species of the parasite responsible for CL, occurs in South America. The majority of diffuse cutaneous leishmaniasis (DCL), characterized by chains of chronic cutaneous lesions along lymphatic vessels, are caused by *L. amazonensis* (46). Although incidence of DCL is rare even in countries where *Leishmania* is endemic, these lesions rarely heal and cause significant scaring and disfigurement (19). Diagnosis of CL is often made upon appearance of typical skin lesions and history of exposure to the vector in endemic regions. More definitive diagnostic techniques include demonstration of the parasite via impression smears, biopsy or culture, as well as polymerase chain reaction (PCR), and serology (40).

Mucocutaneous leishmaniasis (MCL) is a form of CL that is often caused by *L. braziliensis*, *L. panamensis* and less commonly by *L. amazonensis* (53). MCL is characterized by CL-like lesions which involve the nasal mucosa, lips, mouth,

pharynx and/or larynx. These lesions often lead to significant scaring, disfiguring of the face and rarely ever heal (53). In fact, MCL leads to progressive destruction of not only the oronasopharyngeal mucosa, but also the underlying cartilage and can involve the upper airway leading to respiratory obstruction and secondary infections (19).

Visceral leishmaniasis (VL; also known as Kala azar) is typically caused by *L. donovani* in India, Asia and Africa, *L. infantum* in the Mediterranean and *L. chagasi* in South America (59). There are an estimated 500,000 new cases and 50,000 deaths per year due to VL (59). Parasite-infected macrophages disseminate in VL both hematogenously and via lymphatics, infiltrating the bone marrow, liver, spleen and lymph nodes. This dissemination leads to the classical manifestations of disease which include weight loss, hepatosplenomegaly, lymphadenomegaly and eventually massive hemorrhage and edema (59). Patients with VL can also have a dermal manifestation of disease called post Kala-azar dermal leishmaniasis. This form is most commonly associated with *L. donovani* infection and can present years after VL remission (19). Although VL is not considered to be a problem for the human population in the United States, incidence of canine visceral leishmaniasis is increasing within the foxhound population (31, 84).

In the case of CL, most immunocompetent hosts will spontaneously resolve their lesions over time, although some untreated lesions can lead to significant disfiguring scars and social consequences (19). In contrast, treatment is required for MCL, DCL and VL as progression of disease can become life-threatening. Common treatment options to date include pentavalent antimony (PA) compounds, including

sodium stibogluconate and meglumine antimoniate (19, 65). PA is often effective but there are many side effects with treatment, including myalgia, joint stiffness, cardiac arrhythmias, hepatotoxicity, nephrotoxicity and hemolytic anemia (19). PA treatment is considered a more cost-effective option, but resistance to PA has been described in 15% of patients (19). Pentamidine, Miltefosin and Amphotericin B are alternatives to PA and often used when PA fails, although these treatments are considerably more expensive and all have significant side effects (19). Topical treatments can be utilized for CL, including cryotherapy and photodynamic therapy but cost is often prohibitory in endemic regions. Combination therapies are used to enhance efficacy and decrease side effects during *Leishmania* treatment (59). Resistance to treatment along with variable toxic side effects and expense remain important barriers to treatment.

Genetic susceptibility to Leishmania *infection*

Several studies have suggested a familial and/or ethnic susceptibility to leishmaniasis in humans residing in endemic regions of the world (8, 12, 44), suggesting that there could be genetic susceptibility to disease. Researchers have explored this possibility by examining specific genes or gene products that confer disease susceptibility in humans, such as HLA class II polymorphisms (62), decreased nitric oxide production (32), and IL-6 cytokine production (13). Dogs in endemic areas have also been studied, and genetic susceptibilities and resistance mechanisms have been discovered. One group found that two important mutations were found in the NRAMP-1 (SLC11a) gene in susceptible dogs (4). A different

group determined that the Ibizian hound was more resistant to *Leishmania* infection and that this breed can mount a significant cellular immune response to infection as compared to more susceptible breeds (96). It has also been suggested that the Foxhound breed of dogs may also have a genetic susceptibility to visceral leishmaniasis here in the United States (22).

Murine Cutaneous Leishmaniasis

Immune response to L. major versus L. amazonensis

The murine model of infection has been successfully used to characterize the immune response of both susceptible and resistant strains of mice during *L. major* infection. The majority of inbred mouse strains, such as C3H/He, CBA, C57Bl/6 and 129Sv/Ev, are considered resistant to infection and develop local cutaneous lesions that spontaneously resolve over time. The healing phenotype associated with *L. major* infection is characterized by a polarized $CD4^+$ Th1 immune response which activates infected macrophages to kill intracellular pathogens (94). Dendritic cells from resistant mouse strains that are infected with *L. major* produce interleukin (IL)-12 which induces *L. major*-specific $CD4^+$ Th1 to produce interferon (IFN)-γ. IFN-γ will in turn promote IL-12 production and activate inducible nitric oxide synthase in macrophages to produce nitric oxide (NO) (35). NO is a reactive nitrogen intermediate that is toxic to *L. major* and leads to destruction of the parasite within the infected macrophage (56). In contrast, there are a few murine strains, such as BALB/c mice, that develop progressively large, non-healing lesions and are considered susceptible (35, 56). BALB/c mice develop a polarized Th2 immune

response with high levels of IL-4, which is an immune response associated with susceptibility to disease.

Infection of resistant strains of mice with *L. amazonensis* does not produce either a polarized Th1 or Th2 immune response and $CD4^+$ T cells have been shown to disparately promote disease progression and pathology (97). The severity and chronicity of disease caused by *L. amazonensis* is linked to a poor T cell response induced by the parasite (1, 91). Our laboratory has shown that during chronic *L. amazonensis* infection there is decreased production of antigen-specific IFN-γ in the draining lymph node, even after induction of a Th1 response early in infection with IL-12 treatment (103). It has been shown that $CD4^+$ T cells isolated from *L. amazonensis*-infected C3H mice are unable to transition to effector T cells (91). A separate group found that when macrophages were treated with IFN-γ, *L. amazonensis* amastigotes had enhanced replication, indicating a classically described $CD4^+$ Th1 response may not be sufficient (87). These data indicate that *L. amazonensis*-infected mice fail to produce a Th1 polarized effector immune response, and suggests that additional immune responses are necessary for healing this infection.

L. amazonensis *and cross protection with* L. major *infection*

Our laboratory and others have shown that *L. major* infection in a C3H murine model provides protection against subsequent *L. amazonensis* infection (102, 104). Healing of *L. amazonensis* following *L. major* infection is characterized by a 4-6 log decrease in parasite burden as well as resolution of infected footpad lesions.

Significant increases in IFN-γ and IL-12 production have been observed when draining lymph node (DLN) cells from co-infected mice are re-stimulated with *L. major* antigen (102). This suggests that resolution of lesions is correlated with a polarized Th1 response (102). Similar to the cross-protection observed in C3H mice, C57Bl/6 (B6) mice first infected with *L. major* and subsequently challenged with *L. amazonensis* either have small lesions (unpublished observation) or heal the infection (33). These findings indicate that *L. major* infection is able to drive an effective Th1 immune response and aid in healing *L. amazonensis* infection. Our laboratory has developed a co-infection system in which C3H mice are infected with both *L. major* and *L. amazonensis* in the same footpad at the same time. This co-infection results in lesion resolution and a very low parasite load by 10-12 weeks post-infection (30). In contrast, B6 mice do not heal this co-infection and have much higher parasite burdens (30, 33).

C3HeB/FeJ (C3H) versus C57BL/6 (B6) mice and IgG2c antibody production

The B6 inbred mouse strain is the most commonly used strain of mouse for immunologic studies. Many genetically altered mice are bred on a B6 background. B6 mice, as well as C57BL/10, SJL and NOD mice, carry the Igh1-b allele which encodes a portion of the mouse immunoglobulin heavy chain constant region (Igh-C) and produces the antibody isotype IgG2c. In contrast, C3H mice carry the Igh1-a allele and produce IgG2a (60, 68). Immune complexes, or antigen-antibody complexes, have been shown to be associated with visceral leishmaniasis in humans and dogs, and analysis of IgG subclasses have shown that all classes of

IgG are markedly increased with clinical disease (5, 29). A study in dogs determined that levels of IgG2 were higher in dogs that were PCR positive for parasites than those that were negative (88). A separate study found that dogs naturally infected with *Leishmania infantum* had higher IgG2 reactivity than those experimentally infected (51). These findings suggest specific antibody isotypes are important in *Leishmania* infection, but little is known about the function of these antibodies. Interestingly, it has been shown that B cells, the cell type that produces antibodies, in combination with $CD4^+$ T helper cells are required to kill *L. amazonensis* in infected macrophages in vitro (71). The role of B cells in this system appears to be production of antigen-specific antibodies, and more specifically, antigen-specific IgG2a (30).

B cells and Leishmaniasis

Importance of B cells for resolution of *Leishmania* infection

Although B cells and the production of protective antibodies are classically considered to be part of a productive Th2 immune response, some reports in the literature do indicate a protective role for B cells and antibodies during *Leishmania* infection. Scott et. al. showed that blocking B cell production in neonatal C3H mice with an anti-mu antibody impaired T cell-mediated immune responses following *L. major* infection (95). More recently, B cell production of antibody has been shown to be important for phagocytosis of *L. major* by dendritic cells. Without antibodies, infected mice had larger lesion size, higher parasite load, lower IFN-γ production, and a decreased T cell response (109). Other studies have demonstrated the

importance of B cells, but have shown that antibody production actually worsens *Leishmania* infection. Miles, et. al. demonstrated that IgG-negative BALB/c mice infected with *L. major* had smaller lesions with fewer parasites compared to infected mice that had IgG (64). A more recent study showed *L. amazonensis*-infected mice that lacked functional B cells, and therefore antibodies, had delayed onset of disease and developed smaller lesions (106). It has also been described that there were limited infections with both *L. amazonensis* and *L. pifanoi* in the absence of circulating antibodies, and infection of Fc gamma receptor (FcγR) knockout mice resulted in similarly limited lesions (49). FcγR are present on the surface of phagocytic cells and function to bind the Fc portion of antibodies leading to receptor-mediated uptake of opsonized antigens. When FcγRIII knockout mice were infected with *L. mexicana* they did not develop lesions. These mice instead produced a high level of IFN-γ, indicating there is a negative effect when antibodies bind FcγRIII during *L. mexicana* infection (100).

B cells produce many pro-inflammatory and anti-inflammatory cytokines which modulate the immune response. Both IL-1 and IL-6 are produced by B cells (86). IL-1 functions in induction of fever and macrophage activation. IL-6 will also produce fever, along with acute phase proteins and stimulate T and B cell growth and differentiation (45). B cells are reported to produce IL-12 when cultured in a Th1 environment (37). We have shown that B cells produce antigen-specific IL-12 during chronic *L. major* infection, but not during *L. amazonensis* infection (manuscript in preparation). B cells also produce anti-inflammatory cytokines (66) such as IL-10, a potent suppressor of macrophage functions (25, 79) and TGF-β, another anti-

inflammatory cytokine (81). These findings indicate B cells are not only important for antibody production, but also play a role in both cytokine production and polarization of a Th1 immune response. There is evidence that $CD4^+$ T cells are necessary but alone are not sufficient for resolution of *L. amazonensis* infection, and that both $CD4^+$ T cells and B cells are necessary and sufficient for healing (71). The role of both cell types during *L. amazonensis* infection is likely to jointly activate infected macrophages to kill intracellular parasites.

B cell development and activation

B cell development in the bone marrow follows sequential rearrangement and expression of heavy and light-chain immunoglobulin (Ig) genes. Cells that successfully rearrange these genes become immature B cells and express IgM on their surface. Prior to leaving the bone marrow, immature B cells undergo negative selection and receptor editing to eliminate self-reactivity. Immature B cells then migrate to the spleen where they complete maturation and become IgM^+, IgD^+ B cells. Mature B cells seed peripheral lymphoid organs, such as spleen and lymph nodes, where they reside primarily in lymphoid follicles (112). Upon antigen encounter, B cells become activated following cognate interactions with armed helper T cells or undergo T cell-independent activation. T cell-independent activation of B cells occurs when the B cell becomes activated via cross-linking of IgM by certain antigens which trigger IgM synthesis, but neither isotype switching nor memory cell formation occurs (57). T cell-dependent activation begins when there is recognition of specific antigen via the B cell receptor, antigen is internalized,

processed and presented to T helper (Th) cells in the context of major histocompatibility complex (MHC) class II molecules (18). The recognition of peptide:MHC class II complexes by Th cells leads to upregulation of CD154 (CD40 ligand) on the Th cells and engagement of CD40 on B cells. The combination of antigen-receptor binding and CD154-CD40 interactions on B cells will lead to their activation (52). Early after activation, B cells proliferate and differentiate into short term antibody producing cells, most of which secrete IgM. Other activated B cells will serve as germinal center founder cells and initiate the germinal center reaction within follicles.

The germinal center

The germinal center is a site of extensive B cell expansion, isotype switching (a genetic deletion mechanism that allows switching from IgM to downstream IgG, IgA or IgE classes), affinity maturation (mediated by both somatic hypermutation and affinity based selection) and memory B cell formation (99). Germinal centers form within lymphoid follicles of the secondary lymphoid organs, including the spleen, lymph nodes, Peyer's patches and other species-specific lymphoid tissues. Upon stimulation by T cell-dependent antigens, activated B cells can do one of two things: (1) move into extrafollicular zones, proliferate and become short-lived plasma cells, or (2) form B cell follicles in which a small number of cells form germinal centers (3). Short-lived plasma cells do not undergo isotype switching or affinity maturation and thus, the germinal center is necessary for production of high affinity, antigen-specific antibodies. Mature germinal centers are present within 3-4 days following antigen

exposure and have a specific structure (75, 78). They are organized into the dark zone, containing large proliferating B cells which have a very high rate of point mutations during cell division, also known as somatic hypermutation (78). These B cells will then enter the light zone, which is composed of smaller, tightly packed B cells along with follicular dendritic cells and T follicular helper cells (75) (Figure 2). Follicular dendritic cells (fDC) extend long cytoplasmic processes and display immune complexes to B cells. If the newly mutated B cells do not recognize antigen displayed by fDCs they undergo apoptosis; but if they do, they are positively selected and exit the germinal center to become plasma cells or memory B cells (78). fDC's also produce B cell trophic factors and the chemokine CXCL13 which is crucial in organization of the lymphoid follicle (2). T follicular helper (Tfh) cells are also present in the light zone of the germinal center and directly interact with germinal center B cells. Tfh cells are activated T helper cells ($CD4^+$ $CD25^-$) which express an inducible co-stimulator (ICOS), a B cell co-stimulatory molecule, programmed death recptor-1 (PD-1), a negative co-stimulatory molecule and the chemokine receptor CXCR5. CXCR5 is also expressed on germinal center B cells and binds CXCL13 produced by fDC. This chemokine-receptor pairing is necessary for organization of the germinal center light zone (3). Tfh cells also produce the cytokine IL-21 which has been shown to function in B cell proliferation and production of plasma cells (38). The receptor for IL-21 is present on B cells, T cells and NK cells. B cells deficient in the IL-21 receptor have an impaired ability to undergo isotype switching and cannot maintain germinal center organization (24). Tfh cells can also produce IL-10, IL-2, IFN-γ, and IL-4 (38, 61). Tfh cells are

required for maintenance of germinal centers, B cell affinity maturation and production of high affinity antibodies, but not for the formation of the germinal center itself (24, 75). Without Tfh cells, germinal centers do initially form, but rapidly undergo regression (75).

A primary role for the germinal center is to produce B cells committed to antibody production. These are typically isotype switched, high affinity B cells that leave the germinal center and differentiate into long lived plasma cells (LLPC). LLPC typically migrate to the bone marrow where they secrete antibody for extended periods of time (10). Within the germinal center, differentiation into either LLPC or memory B cells (MBC) is determined by the balance of transcription factors within the cell. Expression of B-lymphocyte-induced maturation protein-1 (Blimp-1) and X-box binding protein 1 (XBP-1) will drive plasma cell differentiation (47). In the bone marrow, LLPC require expression of Blimp-1, XBP-1 and interferon regulatory factor 4 (IFR4) for continued survival (47). MBC development is favored by expression of the paired box protein 5 (PAX5) transcription factor. The presence of PAX5 inhibits plasma cell differentiation and its continued expression is necessary for B cell identity (77). The microophthalmia-associated transcription factor (MITF) also prevents plasma cell differentiation and favors MBC production (47).

In addition to T-helper cell dependent activation, B cells can also be activated by a number of toll-like receptor (TLR) agonists. In the mouse, these include TLR2 (lipopeptides), TLR4 (LPS), TLR7/8 (ssRNA) and TLR9 (CpG DNA) ligands. By themselves, TLR agonists can induce activation, proliferation and some differentiation into IgM secreting cells. TLR ligands can also synergize with other

signals (e.g. antigen-receptor binding, CD40 engagement) to further enhance the activation state of B cells or lead to apoptosis (41).

Identification of mature B cell subsets by surface marker expression

All mature B cells in the mouse express B220 and CD19 surface markers. The majority of mature B cells in the peripheral lymphoid organs belong to the follicular B cell subset and can be identified by their $IgM^{intermediate}IgD^{high}CD23^{high}$ phenotype. Upon activation, mature B cells increase surface IgM and progressively lose IgD and CD23. Activated B cells also transiently express CD69 and further upregulate MHC class II, CD80 and CD86 (52). The latter are co-stimulation molecules that foster cognate interactions with Th cells. Upon entering the germinal center, B cells will display peanut agglutinin (PNA) lectin and upregulate CD95 (90). Plasma cells typically lose most B cell-specific markers (B220 and CD19 surface immunoglobulin) but can be identified by CD138 or Syndecan 1 (90). MBC retain B cell markers and in the mouse and can be identified by the expression of CD19, CD38 and the downstream immunoglobulin isotypes (IgG, IgA, or IgE) as well as lack of expression of the early activation marker CD23 and IgM (77).

Macrophages

Macrophage development and phagocytosis

Macrophages are derived from myeloid cells in the bone marrow and when in circulation are considered monocytes. They are resident tissue phagocytic cells that have a role in normal tissue homeostasis and inflammation (28). Macrophages

function to clear apoptotic cellular debris, produce growth factors during homeostasis and have a broad range of pathogen-recognition receptors for clearance of foreign particles (28).

Receptor-mediated phagocytosis

Phagocytosis by macrophages is triggered by the interaction of foreign material with specific phagocytic pathogen-recognition receptors displayed by the cell surface. There are three major pathogen-recognition receptors displayed by the macrophage; complement receptors, mannose receptors and Fc receptors (82). Complement receptors recognize C3b and C3bi fragments which are generated by cleavage of C3 following recognition of pathogen surface components. Macrophage complement receptors include complement receptor (CR)1 (CD35), CR3 (CD11b/CD18) and CR4 (CD11c/CD18). CR1 primarily plays a role in binding foreign particles while CR3 and 4 function in internalization of particles (82). Phagocytosis via complement receptors occurs early in the immune response. Mannose receptors recognize mannose and fucose saccharides present on the capsule of certain bacteria and other pathogens. Fc receptors recognize the Fc portion of immunoglobulin (Ig) and function to recognize and internalize opsonized particles (82). Other receptors on the macrophage also function in particle uptake, including scavenger receptors and lectin-like receptors.

Receptor-mediated phagocytosis of Leishmania

Following inoculation, *Leishmania* promastigotes are phagocytosed by mammalian macrophages and dermal dendritic cells located at the site of the sand fly bite. *Leishmania* parasites have been demonstrated to be taken up into macrophages via multiple receptor-mediated mechanisms. *L. donovani* (15), *L. amazonensis* (89) and *L. mexicana* (83) all can use mannose receptors for entry into macrophages. The parasite can also utilize complement receptors for receptor-mediated cell entry. Binding of CR3 by both *L. major* and *L. amazonensis* has been described, although a major role for these receptors in macrophage infection has not been determined (69) (70). Antibody-opsonized *Leishmania* has also been shown to bind Fc receptors to stimulate receptor update and infection of macrophages (36, 50) (83). Macrophages express Fc gamma receptors (FcγR) on their surface that bind the Fc portion of antibodies; specifically IgG antibodies. After FcγR binds antibody, macrophages can either become activated via downstream signaling by immunoreceptor tyrosine-based activation motifs (ITAMs) or can be inhibited by immunoreceptor tyrosine-based inhibitory motifs (ITIMs). FcγRI and FcγRIII both contain ITAMs, while FcγRIIb contains an ITIM (43). Woelbing et. al. demonstrated that FcγR-deficient mice infected with *L. major* had increased disease susceptibility due to a lack of FcγRI and FcγRIII expression on dendritic cells (109). FcγR-deficient B6 mice resolve infection with *L. mexicana* (a species closely related to *L. amazonensis*) and wild type mice exhibit chronic disease (9). Yang et. al. showed FcγR-activated ERK signaling leads to IL-10 production, and subsequently exacerbated *L. amazonensis* infection (110). These studies emphasize antibody binding, Fc receptor uptake and signaling is important in *Leishmania* infection.

Formation of the phagolysosome

At the site of receptor-recognition of pathogens the macrophage undergoes actin assembly via the Rho family of GTPases, including Cdc42 and Rac1, which control actin assembly via proteins of the Wiskot-Aldrich Syndrome protein (WASP) family. WASP interacts with Arp2/3 forming a multifunctional actin organizer when both CR and Fc receptors are ligand-bound (82). Polymerization of actin filaments is required for protrusion of pseudopodia and formation of the phagocytic cup. Cell membrane lipid products are also necessary for phagocytosis, including phosphatidylinositol-3 kinase (PI3K) and phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P); both of which trigger extension of pseudopods and closure of the phagocytic cup (82). Once foreign particles are phagocytosed they are contained within a membrane-bound endosomal compartment; the phagosome. Early endosomal compartments are distinguished by the presence of Rab5, a small GTPase (34). As the endosome matures it fuses with cytoplasmic lyosomes which contain acid hydrolases to lower the pH of the endosome and function to degrade internalized material (58). Late endosomes display Rab7 and the lysosomal–associated membrane protein-1 (LAMP 1) (34). These membrane-bound compartments function to contain and degrade foreign material as well as regulate protein sorting, trafficking, recycling of receptors and other molecules and cell signaling (34).

Leishmania and the parasitophorous vacuole

The parasitophorous vacuole (PV) is a parasite-containing late endosomal compartment within phagocytic cells (17). Depending on the species of parasite, the PV can be small and harbor only one parasite, as is the case for *L. major* and *L. donovani*, or it can be large and communal, for example, during *L. amazonensis, L. mexicana* and *L. pifanoi* infection (17, 48). PVs contain host membrane components including membrane proteins and phospholipids (39). PVs contain lysosomal enzymes, display late endosomal markers such as Rab7 and LAMP 1 and are formed when parasite-containing phagosomes fuse with late endosomes/lysosomes (17). Formation of PVs often occurs within 30 minutes, following infection with either metacyclic promastigotes or amastigotes in most species, although infection with *L. amazonensis* promastigotes can take longer due to the formation of huge communal PVs (7). The ultrastructure of the PV during *L. major* infection revealed that a single amastigote is present within a small, tightly membrane-bound vacuole (14). In contrast, multiple *L. amazonensis* amastigotes are present within a single, large membrane-bound PV and attachment of the amastigote membrane is limited to a small portion of the PV membrane (14).

Macrophage killing mechanisms:

Respiratory burst

During and immediately following macrophage phagocytosis the respiratory burst is triggered. This event is marked by an increase in superoxide generated within the phagocytic cup and newly formed phagosome. This burst is primarily activated

through Fc receptor-ligand binding at the macrophage surface (82). Superoxide is produced by the NADPH oxidase enzyme complex composed of 5 subunits which are either membrane bound or cytosolic. $P22^{phox}$ and $gp91^{phox}$ (together called cytochrome b558) are the two membrane-bound components present on cytoplasmic vesicles that fuse with the plasma membrane during phagocytosis (82). The three cytosolic components include $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ and are present as a complex within resting cells (76). Two Ras-related small GTP-binding proteins are also required; Rap 1 and Rac1, which function to regulate the NADPH complex assembly (82). At the time of phagocytosis $p47^{phox}$ becomes phosphorlyated by protein kinases and GTP binds to Rac1. This phosphorylation causes trafficking of the three cytosolic subunits to the membrane where they associate with cytochrome b558 and thus create assembled NADPH oxidase (82) (Figure 3). The $p47^{phox}$ subunit plays a key role in this activation, as it is the phosphorylated subunit, while $p67^{phox}$ appears to be the subunit that is needed to link cytosolic subunits to membrane-bound components (76). The role of $p40^{phox}$ during superoxide generation and complex assembly is controversial. Recently it has been described that the $p40^{phox}$ subunit primarily functions to regulate Fc gamma receptor-induced NADPH oxidase activity, not assembly of the complex. This subunit stimulates superoxide production via a phosphatidylinositol-3-phosphate (PI3P) signal following phagocytosis and thus is a key subunit in generation of superoxide via FcγR activation (101). FcγR activation will activate class I PI3 kinases, generating phosphatidylinositol 3,4,5 triphosphate (PI(3,4,5)P) and phosphatidylinositol 4,5 bisphosphate (IP(3,4)P) on the phagosome cup and class III PI3K, which will

produce PI3P on internalized phagosomes (111). PI3P will bind $p40^{phox}$ and stimulate oxidase activity, which has been described in both neutrophils and macrophages (23, 101). Interestingly, a recent study showed that superoxide, along with nitric oxide, was necessary within *L. amazonensis*-infected macrophages to kill the parasite (72).

Nitric Oxide

Nitric oxide (NO) is produced by classically-activated macrophages and functions to kill intracellular pathogens. Inducible nitric oxide synthase (iNOS) is the catalyst that generates NO and is one of three isoforms of nitric oxide synthase which generates nitric oxide from L-arginine (42). iNOS is primarily expressed in macrophages and neutrophils. Following production of NO, which interacts with water and oxygen, there is generation of multiple reactive nitrogen intermediates, including NO_2^-, NO_3^-, N_2O_2 and peroxynitrite (42). These reactive intermediates are directly toxic to intracellular pathogens, including *Mycobacterium* and *Leishmania major* (42, 54). NO functions to kill pathogens via multiple mechanisms including S-nitrosylation of proteins, tyrosine nitration, deprivation of iron, inhibition of DNA synthesis, DNA oxidation, DNA degradation, and oxidation of lipids (reviewed in (20). iNOS is activated by multiple pathogen-derived ligands, including lipopolysaccharide (LPS) and mycobacterial lipoarabinomannins (42). iNOS is also strongly activated by Th1 cytokines, specifically IFN-γ. IFN-γ binds its receptor and rapidly activates the Janus tyrosine protein kinases (JAK)-STAT signaling pathway (16). JAK1/2 is activated by tyrosine phosphorylation and allows release of STAT1

so it can form a homodimer, translocate to the nucleus and bind IFN-γ-activated sequence elements, including IFN response elements (16) (Figure 4). It has been demonstrated that IFN response elements flank the murine iNOS gene and transcriptional induction of iNOS by IFN-γ depends on JAK-STAT activation (16).

Leishmania *evasion of macrophage killing mechanisms*

Leishmania species resist multiple macrophage killing mechanisms once phagocytosed. Lipophosphoglycan (LPG), a parasite surface glycolipid has been shown to inhibit macrophage-produced reactive oxygen species during *L. major* infection (98). LPG from *Leishmania donovani* was also shown to block NADPH oxidase assembly at the membrane of the phagolysosome (55). *L. donovani* has been shown to inhibit protein kinase C (PKC) activity; a signaling cascade necessary for NADPH oxidase assembly and superoxide production (48). Opsonized *L. amazonensis* entry into cells is Rac-1 independent (67), and Rac-1 activation is required for assembly of the NADPH oxidase complex. Therefore *L. amazonensis* likely avoids induction of superoxide during Fcγ receptor-mediated phagocytosis (67). Although Fcγ receptor-mediated phagocytosis has been shown to induce superoxide production, complement receptor-mediated uptake utilizes a distinctly different signaling mechanism, Rho, instead of Rac therefore NADPH oxidase assembly does not occur (11). This means that parasites taken up via complement receptors likely do not trigger production of superoxide. The NADPH oxidase complex did not form on PVs during *L. pifanoi* amastigote infection, but it did form when there was infection with *L. pifanoi* promastigotes (85). In this case it appeared

that there was an unstable association of $gp91^{phox}$ and $p22^{phox}$ (48). Finally, some species of *Leishmania*, including *L. chagasi*, *L. tropica* and *L. donovani* have been shown to produce superoxide dismutase, an enzyme that neutralizes superoxide into water and oxygen (63, 73, 80). *Leishmania* infection can also inhibit NO production via immunomodulation. *L. amazonensis* can induce secretion of IL-10 and TGF-β which will inhibit the induction of iNOS and production of nitric oxide (107). Several studies have also proven that *Leishmania* can inhibit IFN-γ-activation of JAK1 and 2 and Stat 1 signaling, thus inhibiting iNOS activation (6, 26, 74). *Leishmania* can inhibit production of IL-12 from infected macrophages, thus dampening the activation of a productive Th1 immune response (reviewed in (48)). A productive immune response can also be hampered by some species of *Leishmania* infection by causing internalization and subsequent degradation of MHC class II proteins needed for antigen presentation and initiation of a robust, productive T cell response (reviewed in (7)). All of these mechanisms provide for parasite evasion of the macrophage killing response and intracellular persistence.

Overview of thesis and project objectives

The objectives of this dissertation were to (1) determine whether phenotypic differences in specific cell type(s) leads C3H mice to heal a co-infection with *L. major* and *L. amazonensis* while B6 do not, (2) determine the phenotypic and/or functional difference between B cells from co-infected C3H mice and co-infected B6 mice, (3) to determine the signaling pathways triggered in *L. amazonensis*-infected macrophages that can lead to generation of both superoxide and nitric oxide. These

findings significantly advance our knowledge concerning the importance of B cells in the cell-mediated anti-*Leishmania* immune response, provide a better understanding for murine strain differences in response to *Leishmania* co-infection and allow us to discover how to promote a productive immune response against *L. amazonensis*.

The findings presented here will not only help us understand the role of B cells in chronic *Leishmania* infection, but also will allow us to gain insight into the specific immune factors needed for healing of *L. amazonensis* infection in vivo. These studies will assist in determining what is necessary for protective vaccination and treatment of this disease. By identifying immunologic differences between two different mouse strains, we hope to be able to apply these findings to the understanding of genetic susceptibility to disease in other animals, specifically dogs, as well as humans. Identification of the roles of both $CD4^+$ T cells and B cells in the role of macrophage activation and killing of *L. amazonensis* will also aid in our understanding of a productive immune response. Defining the pathways of macrophage activation by both of these cell types will allow us to determine what factor(s) are necessary to resolve chronic infections with *L. amazonensis*. Overall, these findings will aid us in identifying factors necessary for resolution of *L. amazonensis* infection and why different mouse strains respond differently to infection. These concepts can then be applied to determining how people respond to *Leishmania* infection, as well as other persistent infections.

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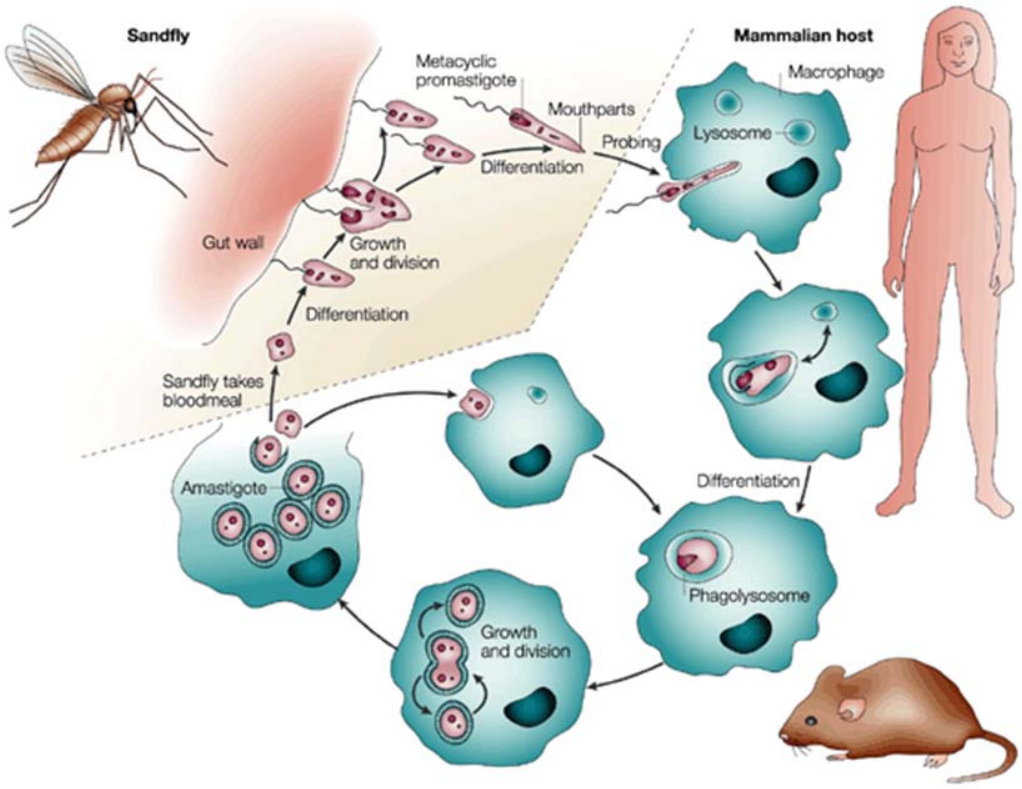

Figure 1. Life cycle of *Leishmania major* infection. *Leishmania* parasites are transmitted by the bites of infected female sand flies, which inject a small number of infectious-stage, metacyclic promastigotes into the skin. These forms are opsonized efficiently by serum components and taken up by macrophages, where they reside in phagolysosomes and transform into replicating amastigotes. Infected macrophages are taken up by sand flies during blood feeding; they are lysed in the fly midgut, releasing parasites that transform into rapidly dividing, non-infectious-stage promastigotes. These forms undergo a process of attachment to the midgut wall, release and anterior migration that is accompanied by their differentiation to non-dividing, metacyclic promastigotes that can be transmitted when the sand fly takes another blood meal. Figure and legend reprinted by permission from Macmillian Publishers Ltd: *Nature Reviews Immunology*, Sacks, D and N. Noben-Trauth. 2(11): 845-58, copyright 2002.

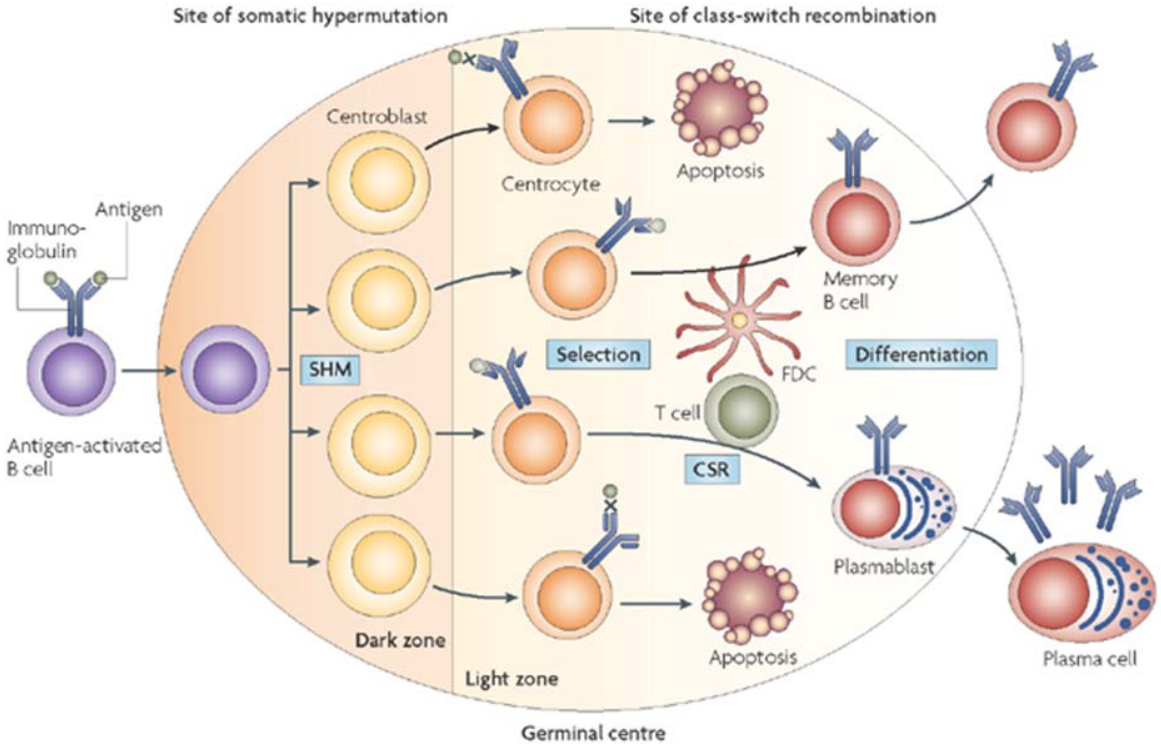

Figure 2. Organization of the germinal center. Antigen-activated B cells differentiate into centroblasts that undergo clonal expansion in the dark zone of the germinal centre. During proliferation, the process of somatic hypermutation (SHM) introduces base-pair changes into the V(D)J region of the rearranged genes encoding the immunoglobulin variable region (IgV) of the heavy chain and light chain; some of these base-pair mutations lead to a change in the amino-acid sequence. Centroblasts then differentiate into centrocytes and move to the light zone, where the modified antigen receptor, with help from immune helper cells including T cells and follicular dendritic cells (FDCs), is selected for improved binding to the immunizing antigen. Newly generated centrocytes that produce an unfavourable antibody undergo apoptosis and are removed. A subset of centrocytes undergoes immunoglobulin class-switch recombination (CSR). Cycling of centroblasts and centrocytes between dark and light zones seems to be mediated by a chemokine gradient, presumably established by stromal cells in the respective zones (not shown). Antigen-selected centrocytes eventually differentiate into memory B cells or plasma cells. Figure and legend reprinted by permission from Macmillian Publishers Ltd: *Nature Reviews Immunology*, Klein, Ulf and Riccardo Dalla-Favera. 8: 22-33, copyright 2008

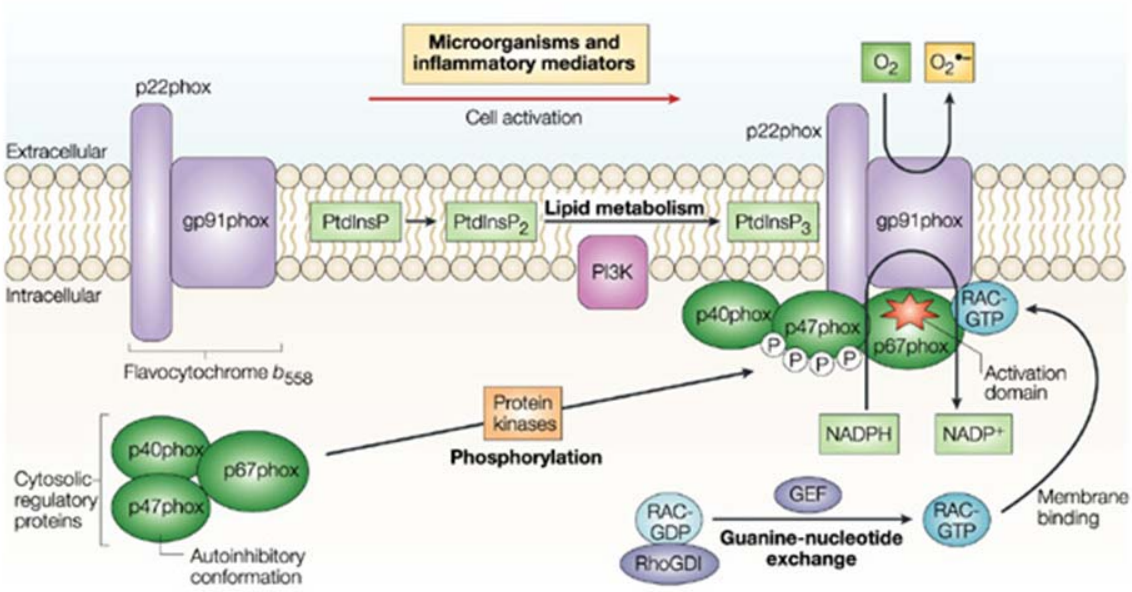

Figure 3. Activation of reactive oxygen species (ROS) generation by assembly of Phox regulatory proteins in phagocytes. Activation of the gp91phox system occurs by at least three signalling triggers that result in the assembly of cytosolic regulatory proteins (p40phox, p47phox and p67phox) with flavocytochrome *b*558 (comprised of the membrane-associated catalytic subunit gp91phox plus p22phox). These triggers involve protein kinases, lipid-metabolizing enzymes and nucleotide-exchange proteins that activate the GTPase RAC. Protein kinases, including protein kinase C and AKT, catalyse many phosphorylations of the autoinhibitory region (AIR) of p47phox, releasing its binding to the bis-SRC-homology 3 (SH3) domain, allowing p47phox to bind to p22phox. This also relieves inhibition of the Phox homology (PX) domain of p47phox, allowing binding to lipids. Because p47phox also binds to p67phox, it has been described as an organizer protein. Phosphatidylinositol 3-kinase (PI3K) and phospholipase D produce 3-phosphorylated phosphatidylintositols (PtdInsP) and phosphatidic acid, respectively, providing lipids to which the p47phox and p40phox PX domains bind. RAC is post-translationally modified with a carboxy-terminal hydrophobic geranyl-geranyl group. In RAC-GDP, this group is masked by the inhibitory protein RhoGDP-dissociation inhibitor (RhoGDI), maintaining RAC in the cytosol. Activation of exchange factor(s) triggers GTP binding, resulting in conformational changes in RAC that promote dissociation from RhoGDI and membrane association through the geranyl-geranyl lipid. The conformational change also promotes RAC binding to the tricodecapeptide (TPR) region of p67phox, helping to assemble the active complex.Figure and legend reprinted by permission from Macmillian Publishers Ltd: *Nature Reviews Immunology*, Lambeth, J. David. 4; 181-189, copyright 2004.

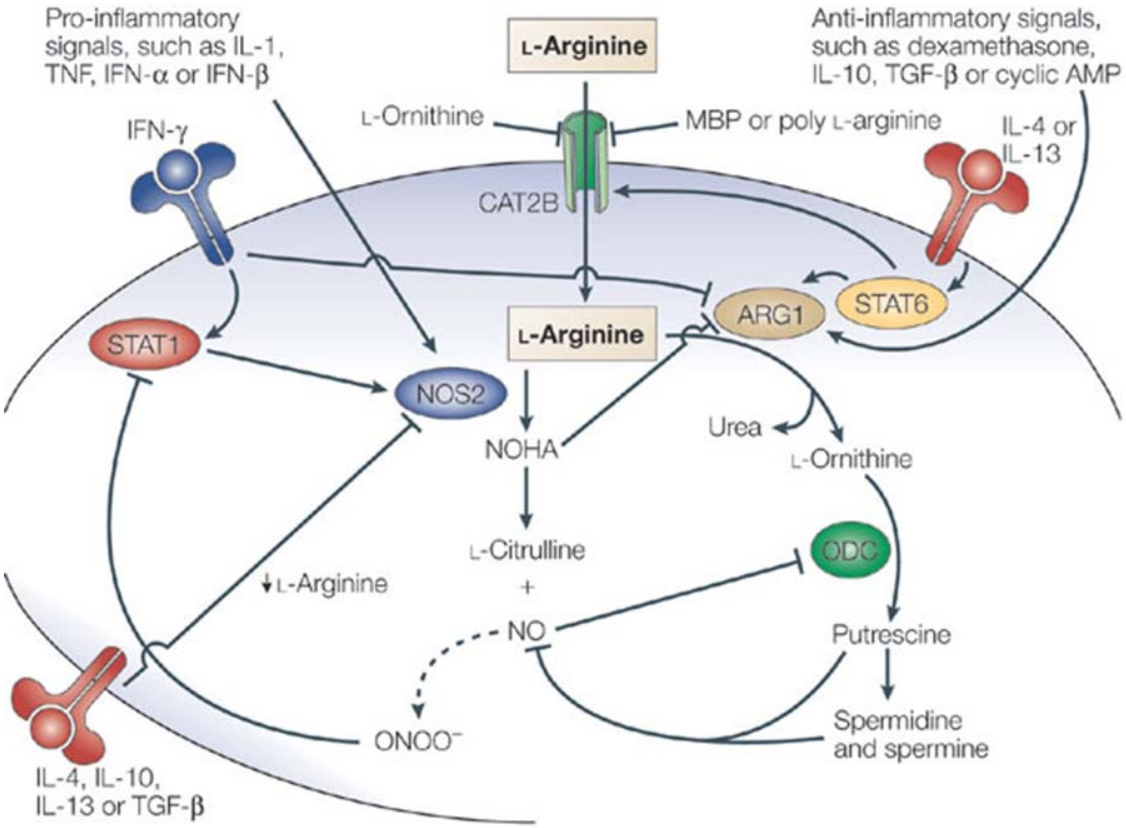

Figure 4. Cytokine-dependent and metabolic regulatory circuits that affect the inducible enzymes arginase 1 and nitric-oxide synthase 2. The T helper 1 (TH1) cytokine interferon- (IFN-) and the TH2 cytokines interleukin-4 (IL-4) and IL-13 are the main inducers of nitric-oxide synthase 2 (NOS2) and arginase 1 (ARG1), respectively. Pro-inflammatory signals (such as IL-1, tumour-necrosis factor (TNF), IFN-γ and IFN-γ) and anti-inflammatory signals (such as IL-10, transforming growth factor- (TGF-β), cyclic AMP and dexamethasone) can contribute to regulate the final balance between NOS2 and ARG1 activity. Moreover, ARG1 and NOS2 directly activate several biochemical circuits that negatively regulate each other. Low extracellular L-arginine concentration, overexpression of ARG1 or reduction of the capacity for L-arginine uptake can all decrease intracellular L-arginine levels (L-arginine) and halt the translation of mRNA encoding NOS2, thereby reducing the activity of NOS2. Molecules and products of L-arginine metabolism function in the cytosol but can also be released extracellularly. Nitric oxide (NO), *N*G-hydroxy-L-arginine (NOHA), L-ornithine, polyamines (including putrescine, spermidine and spermine), *N*G-monomethyl-L-arginine monoacetate and even ARG1 itself can be found in the extracellular space. ONOO-, peroxynitrite; STAT, signal transducer and activator of transcription. Figure and legend reprinted by permission from Macmillian Publishers Ltd: *Nature Reviews Immunology*, Bronte, Vincenzo and Paola Zanovello. 5; 641-654, copyright 2005.

Chapter 2

A DEFICIENCY IN THE B CELL RESPONSE OF C57Bl/6 MICE CORRELATES WITH LOSS OF MACROPHAGE-MEDIATED KILLING OF *LEISHMANIA AMAZONENSIS*

A modification of the paper published in International Journal of Parasitology[1]

Katherine N. Gibson-Corley [2, 4], Paola M. Boggiatto [2], Rami M. Mukbel [3], Christine A. Petersen [2, 5], Douglas E. Jones [2, 5, 6]

Abstract

Infection of C3HeB/FeJ and C57BL/6 mice with *Leishmania major* stimulates a healing cell-mediated immune response, while *Leishmania amazonensis* infection leads to chronic disease. Here we show C3HeB/FeJ mice co-infected with both species of *Leishmania* heal, while co-infected C57BL/6 mice do not. Using an in vitro killing assay we determined B cells from infected C57BL/6 mice are ineffective in promoting parasite killing compared to B cells from infected C3HeB/FeJ mice. Furthermore, infected C57BL/6 mice produce less antigen-specific antibodies as compared to infected C3HeB/FeJ mice. These findings suggest B cells play a required role in the cell-mediated immune response against *L. amazonensis*.

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Introduction

Leishmaniasis is a zoonotic, vector-borne disease caused by an obligate intracellular protozoan parasite of the genus *Leishmania*. Infection of C3HeB/FeJ (C3H) mice with *Leishmania amazonensis (La)* leads to chronic disease with large non-resolving cutaneous lesions and high parasite loads (7). The *La*-induced immune response is neither a T helper 1 (T_H1) or a T helper 2 (T_H2) response, as evidenced by unpolarized $CD4^+$ T cells that fail to efficiently produce either IFN-γ or IL-4 and by dendritic cells that produce little IL-12 (1, 5, 6, 14). Experimental evidence derived using *Leishmania major (Lm)* indicates that protection from these parasites requires establishment of a polarized T_H1 immune response characterized by production of IL-12 and subsequent activation of IFN-γ -producing $CD4^+$ T cells (15). More recent studies demonstrated that *La*-specific T_H1 $CD4^+$ T cells were ineffective in killing *La* in vivo and did not promote lesion resolution (13, 18). However, our laboratory and others have shown that T_H1 immunity associated with *Lm* infection provided significant protection against subsequent *La* infection (2, 17, 19).

Similar to the cross-protection observed in C3H mice, C57BL/6 (B6) mice first infected with 1 x 10^4 *Lm* and subsequently challenged with *La* also heal the infection, but interestingly, these mice do not heal a simultaneous infection with both *Lm* and *La* (2). To better understand the cellular mechanism underlying the productive healing response provided during co-infection of C3H mice and not B6 mice, we performed a simultaneous co-infection with *Lm* and *La* in both the C3H and B6 mouse models.

Materials and Methods

Parasites. La (MHOM/BR/0016/LTB) and *Lm* (MHOM/IL/80/Friedlin) promastigotes were grown in complete Grace's Insect medium (Atlanta Biologicals, Lawrenceville, GA) to stationary phase, harvested, washed in endotoxin-free PBS (Cellgro, Herdon, VA) and diluted to a concentration of 1 x 10^8 parasites per milliliter.

Mice. Female C3HeB/FeJ mice (6-8 weeks of age) were bred in-house in a specific pathogen-free environment. The Institutional Animal Care and Use Committee at Iowa State University approved all protocols involving animals. Female C57BL/6 mice of the same age were obtained from Jackson Laboratories (Bar Harbor, Maine).

In vivo co-infection. Mice with a single infection were inoculated with 5 x 10^6 *La* or *Lm* stationary phase promastigotes, while co-infected mice were inoculated with 2.5 x 10^6 stationary phase *Lm* promastigotes plus 2.5 x 10^6 *La* promastigotes, totaling 5 x 10^6 parasites in the left hind footpad. Mice were infected for 12 weeks with weekly monitoring of lesion size. At 12 weeks p.i. the mice were euthanized and parasite quantification in the infected footpad was performed using limiting dilution.

In vitro killing assay. Bone marrow-derived macrophages were derived from naïve C3H and B6 mice as described previously (12). Briefly, after 6 days in culture BMDM were harvested, counted, and 5 x 10^5 macrophages were added into the bottom compartment of 24 well transwell plates (Corning Costar, NY), each containing a coverslip (Fisher Scientific, Hanover Park, IL) in complete tissue culture media (CTCM). Macrophages were infected with *La* amastigotes at a 3:1 parasite to macrophage ratio. Popliteal lymph nodes were harvested from

C3H or B6 mice infected for 4 weeks with *Lm*. $CD4^+$ T cells (Miltenyi Biotech, Auburn, CA) or $CD19^+$ B cells (MagCellect, R&D system, Minneapolis, MN) were purified via depletion using an autoMACS^{TM} separator (Miltenyi Biotech, Auburn, CA). The $CD4^+$ T cells and B cells were added to the top compartment of 0.4 μm diameter transwell along with freeze-thawed *Lm* promastigote antigen, as indicated in Figure 2. When allogeneic B cells were assessed they were placed separately into the upper chamber and the syngeneic $CD4^+$ T cells and macrophages were placed together in the bottom chamber, necessitating the use of BMDM from both C3H (Figure 2A) or B6 (Figure 2B) mice. All experimental conditions were performed in duplicate. Coverslips were harvested after 5 days of culture, fixed with 100% methanol and stained with the HEMA 3 stain set (Fisher Scientific, Hanover Park, IL). Data analysis was performed by counting the coverslips via light microscopy and examining three areas at 100x magnification. In each area, 100 macrophages were examined assessing the number of infected macrophages and the number of parasites per 100 infected macrophages.

Statistical Procedure. Statistical analysis was performed using Statview (SAS, Cary, NC). When treatment groups were compared, the data was analyzed with ANOVA and Scheffe pair-wise comparisons. When two treatment groups were compared, data was analyzed using an unpaired student's t-test. Differences were considered significant when $P < 0.05$.

Results

Simultaneous co-infection with both *Leishmania major (Lm)* and *Leishmania amazonensis (La)* allows for lesion resolution in C3H but not B6 mice.

After co-infection with *La* and *Lm*, C3H mice had negligible footpad lesions by 12 weeks p.i., similar to infection with *Lm* alone (Figure 1A). Parasite load from infected footpads was between 10^2 and 10^3 parasites (Figure 1C). In contrast, co-infected B6 mice developed large footpad lesions that persisted for the 12 week period (Figure 1B) and parasite burdens were 10^6 to 10^7 parasites per footpad, similar to infection with *La* alone (Figure 1C). Therefore, co-infection with *Lm* leads to decreased footpad lesion size during concurrent *La* infection in C3H mice, but not in B6 mice.

B cells isolated from L. major-infected C57Bl/6 mice do not promote killing of L. amazonensis in vitro.

To better understand the underlying cellular mechanism of the difference observed after co-infection of C3H versus B6 mice, we employed an in vitro assay developed in our laboratory (12) to assess which immune cell-types, derived from the draining lymph node (DLN) of *Lm*-infected C3H mice, are required to deplete *La* infection. This assay utilizes bone marrow-derived macrophages (BMDM) from C3H mice infected with *La* amastigotes for 24h in vitro. These infected macrophages are then co-cultured with DLN cells harvested from C3H mice that had been infected with *Lm* for 4 weeks, the point at which footpad lesions are resolving. Our previous studies determined that although antigen-specific $CD4^+$ T cells are required, they are not sufficient to promote

macrophage killing of intracellular *La*. Antigen-specific $CD19^+$ B cells and their antibodies were found to be additional necessary components for macrophage killing of intracellular *La* amastigotes in this assay (12).

Using transwell chambers we showed that direct contact between infected macrophages and the lymphocytes was not required for the observed parasite depletion, allowing experiments with syngeneic cells. However, although B cells could be isolated to the upper chamber, $CD4^+$ T cell function required cell contact with either the macrophage in the lower chamber or the B cell in the upper chamber. This fact precludes the ability to test syngeneic macrophages and B cells with allogeneic $CD4^+$ T cells without the confounding influence of a mixed lymphocyte response. Based on these findings and the fact that co-infected B6 mice do not heal (Figure 1B), we hypothesized that $CD4^+$ T cells and B cells from the DLN of *Lm*-infected B6 mice would not be able to induce macrophages to kill *La* in our in vitro co-culture assay.

Figure 2 shows $CD4^+$ T cells and B cells isolated from *Lm*-infected B6 mice do not reduce the percentage of infected macrophages in vitro when compared to the same cells isolated from *Lm*-infected C3H mice. This phenomenon is not affected by the mouse strain from which the macrophages are derived (Figure 2A, first and second bars; Figure 2B, first and third bars). Furthermore, B cells from C3H mice promoted killing of *La* even in combination with $CD4^+$ T cells from B6 mice (Figure 2B, second bar). In contrast, B cells from B6 mice did not promote killing even with $CD4^+$ T cells isolated from C3H mice (Figure 2A, third bar). These results demonstrate that the inability of B6 mice to control the co-infection tracks with an inability of B6 B cells to aid in the killing of *La* in our in

vitro killing assay system. Thus B cells from *Lm*-infected B6 mice are not functionally equivalent to those from *Lm*-infected C3H mice.

C57Bl/6 mice produce low levels of *Leishmania major*-specific antibodies in vivo.

Based on the conclusion that B cells from C3H and B6 mice did not demonstrate equivalent responses in vitro, we wanted to determine if there were differences in their B cell responses in vivo. Therefore we compared parasite-specific total IgG and IgG2a or IgG2c (10) antibody production during infection of C3H or B6 mice, respectively. ELISA was performed on serum for antibody isotype titers. All samples from both C3H and B6 mice were positive for total IgG, IgG2a or IgG2c, at 1:10,000 dilutions at 5 and 12 weeks post-infection, indicating readily detectable serum antibodies. Western blot analysis was performed with serum (1:25 dilution) from mice infected for 5 or 12 weeks hybridized against freeze-thawed *La* and *Lm* stationary phase promastigote parasite antigen. C3H mice infected with *Lm* alone or co-infected produced readily demonstrable parasite-specific total IgG and the T_H1-associated IgG2a isotype to both *La* and *Lm* antigens (Figure 3a, b) at both 5 and 12 weeks p.i. No parasite specific IgG2a was detected after *La* infection at 5 weeks with a small amount seen at 12 weeks post-infection (Figure 3b). In contrast, B6 mice produced only small amounts of parasite-specific total IgG (Figure 3a) and almost undetectable amounts of the T_H1 associated IgG2c isotype, which was *Lm*-specific even after co-infection (Figure 3b). These results were recapitulated using amastigote-derived antigen preparations for western blot analysis (data not shown).

Discussion

Our results support the premise that B cells play a necessary role in an effective T_H1-mediated immune response towards *La* and that the inability of B6 mice to heal a co-infection correlates with a defect in the B cell response, rather than an exclusive defect in the $CD4^+$ T cell response. Although B cells and the production of protective antibodies are classically considered part of a productive T_H2 immune response, several reports indicate a protective role for B cells and/or antibodies during *Leishmania* infection. Recently, B cell production of antibodies has been shown to be important for phagocytosis of *Lm* by dendritic cells. Without antibodies, *Lm*-infected mice had larger lesion sizes, higher parasite loads, low IFN-γ production, and a decreased T cell response, indicating antibodies supported resolution of the infection (21). In contrast, other studies have indicated the importance of B cells in enhancing immunopathology during *Leishmania* infection. Miles et al. showed that in the absence of IgG, *Lm*-infected BALB/c mice had smaller lesions with fewer parasites as compared to infected wild-type mice (11). Recently, another study using mice that lack functional B cells and antibodies, showed *La*-infected mice had a delayed onset of disease and developed small lesions (20). Kima et. al. also described limited infections with both *L. amazonensis* and *L. pifanoi* in the absence of circulating antibodies, and infection of Fc gamma receptor (FcγR) knockout mice resulted in similarly limited lesions (9). In addition, work by Thomas and Buxbaum showed FcγRIII knockout mice were resistant to *L. mexicana* infection. These mice healed their lesions with a high level of IFN-γ production, indicating antibody stimulation of FcγRIII was detrimental during *L. mexicana* infection (16).

Our results are not in disagreement with these studies. Our previous and current work suggests that any effective immune response against *La*, in addition to other factors, must include a productive antigen-specific B cell response that can promote macrophage-mediated parasite killing. Following inoculation, *Leishmania* promastigotes are phagocytosed by mammalian macrophages and dendritic cells located at the site of infection. Macrophages express FcγR on their surface that bind the Fc portion of antibodies; specifically IgG antibodies. After FcγR-antibody binding, macrophages can become activated to produce effector molecules including NADPH-oxidase dependent superoxide production via immunoreceptor tyrosine-based activation motifs (ITAMs) (4). A recent study demonstrated that superoxide generation was a required factor in killing *La* in human cases of chronic disease (8). In the C3H mouse model, our laboratory has shown that B cells and IgG antibodies along with $CD4^+$ T cells from an established *Lm* infection are necessary for providing effective stimulation to macrophages for superoxide dependent killing of *La* (12). Neutralization of IgG2a in vitro negates killing of *La* within infected macrophages (Supplemental data). However, replacement of C3H B cells with serum from *Lm*-infected C3H mice is not sufficient for killing of *La* in vitro (unpublished observations).

Our results indicate B6 mice produce detectable but low levels of *Lm* parasite-specific total IgG and IgG2c antibodies during infection, as compared to total IgG and parasite-specific IgG2a produced by C3H mice. This overall low level of parasite-specific IgG2c antibody production may result in inadequate stimulation of Fcγ receptors, limiting superoxide production. Overall, our findings indicate that antibodies are necessary, but not sufficient, for killing *La* and that antibodies are just one of several critical immune components required for killing.

The known complexities of antigen-antibody and FcγR-mediated immustimulatory vs. immunoregulatory mechanisms (reviewed in (3)) suggests that simple serum or B cell transfer experiments may not adequately recapitulate all of the immune components required for killing *La* either in vivo or in vitro, consistent with our own unpublished observations.

In conclusion, the inability of B6 mice to heal a co-infection of *Lm* and *La* correlates with a defect in the B cell response, rather than the $CD4^+$ T cell response, as defined by our in vitro killing assay. These results suggest that both effective $CD4^+$ T cells and B cells are required for a protective, healing, cell-mediated immune response to *La*.

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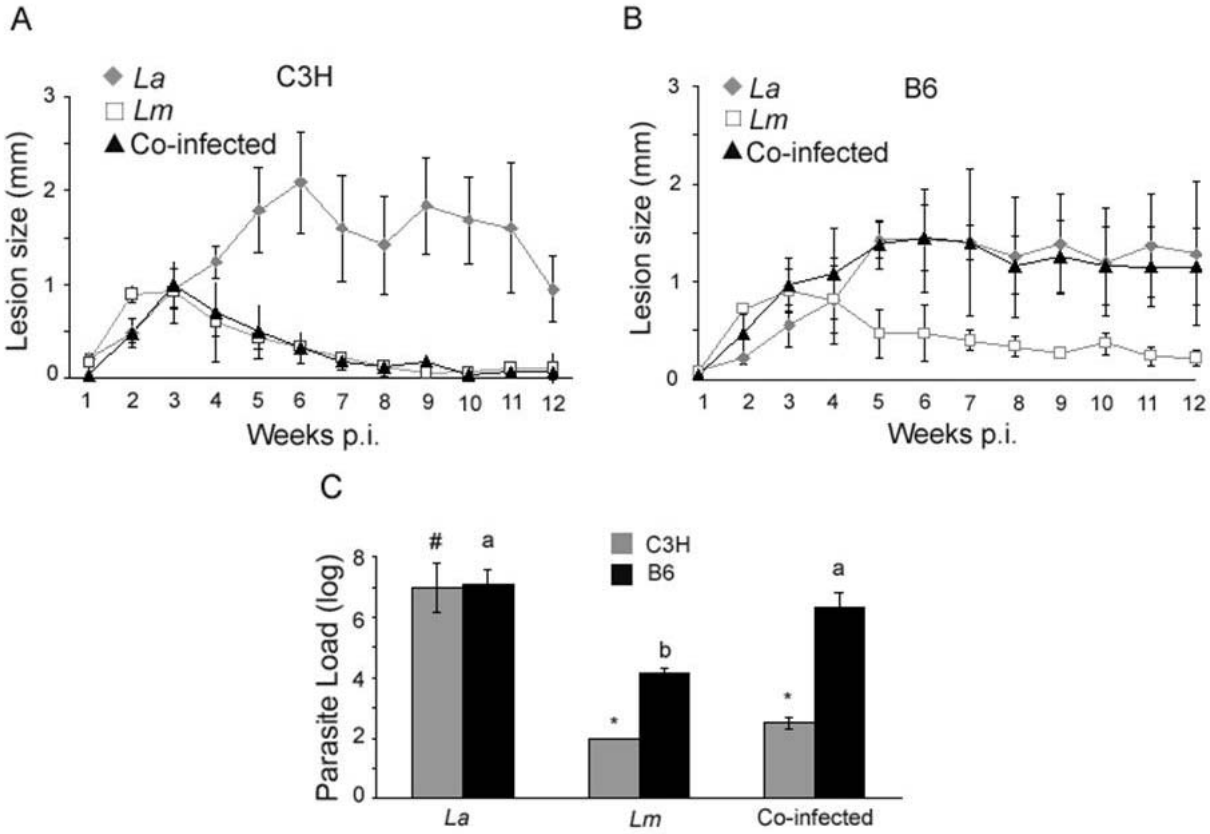


Figure 1. Simultaneous co-infection with both *Leishmania major (Lm)* and *Leishmania amazonensis (La)* allows for lesion resolution in C3H but not B6 mice. A. Lesion size of co-infected C3H mice was significantly different from C3H mice infected with *La* alone (grey diamonds) ($p < 0.001$), while **B.** co-infected B6 mice were significantly different from the B6 mice infected with *Lm* alone (open squares) ($p < 0.001$). Lesion size was determined by measuring the infected footpad and comparing that to the non-infected footpad. Repeated measure ANOVA was used for statistical analysis. Results are representative of three separate experiments. **C**. The number of parasites in the lesions of co-infected C3H and B6 mice. Infected footpads were harvested and parasite suspensions were serially diluted in Complete Grace's medium and incubated at 27°C for 5 to 7 days. Different symbols (*, #) represent a statistically significant difference ($p < 0.001$) within the C3H infection groups and different letters (a, b) represent significant differences ($p < 0.001$) within the B6 infection groups. ANOVA and Scheffe pair-wise comparisons using Stat View software were used for statistical analysis. Results are the mean and SE from three separate experiments.

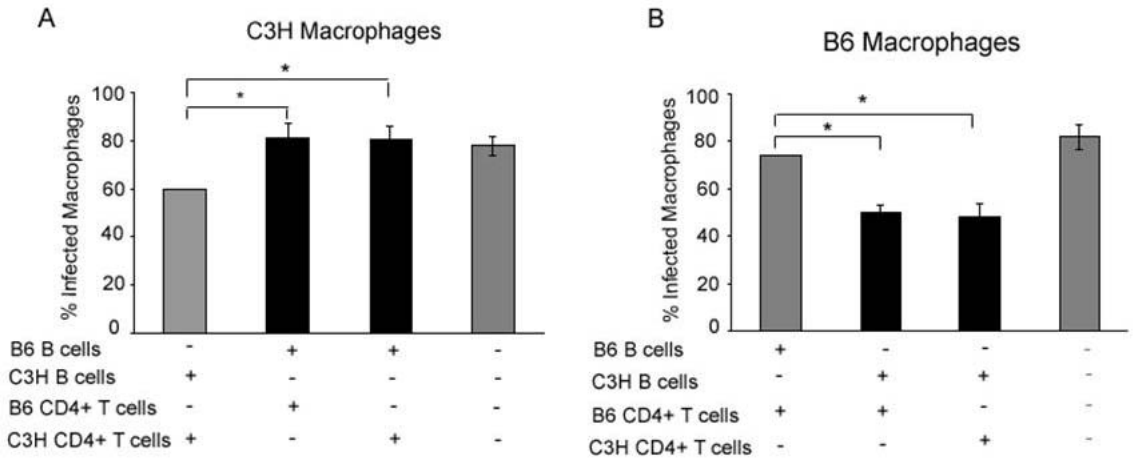


Figure 2. B cells isolated from *Leishmania major (Lm)*-infected C57BL/6 (B6) mice do not promote killing of *Leishmania amazonensis (La)* in vitro. A. $CD19^+$ B cells and $CD4^+$ T cells, purified from the draining lymph node (DLN) of C3H or B6 mice infected with *Lm* for 4 weeks, were placed into the upper chamber of a transwell plate with *La*-infected C3H-bone marrow-derived macrophages (BMDM) in the lower chamber as indicated above (1st and 2nd bars, respectively). B cells from B6 mice were isolated to the upper chamber and $CD4^+$ T cells from C3H mice were placed in the bottom chamber (3rd bar). All wells contained *Lm* freeze-thawed antigen and were incubated for 5 days at 34°C. Black shading indicates the presence of B cells from B6 mice. **B.** Same as A except with BMDM derived from B6 mice and B cells from C3H mice were isolated to the upper chamber (2nd bar) as indicated. Black shading indicates the presence of B cells from C3H mice. * represent statistically significant differences ($p < 0.001$) as determined by ANOVA and Scheffe pair-wise comparison. Results are the mean and SE from three separate experiments.

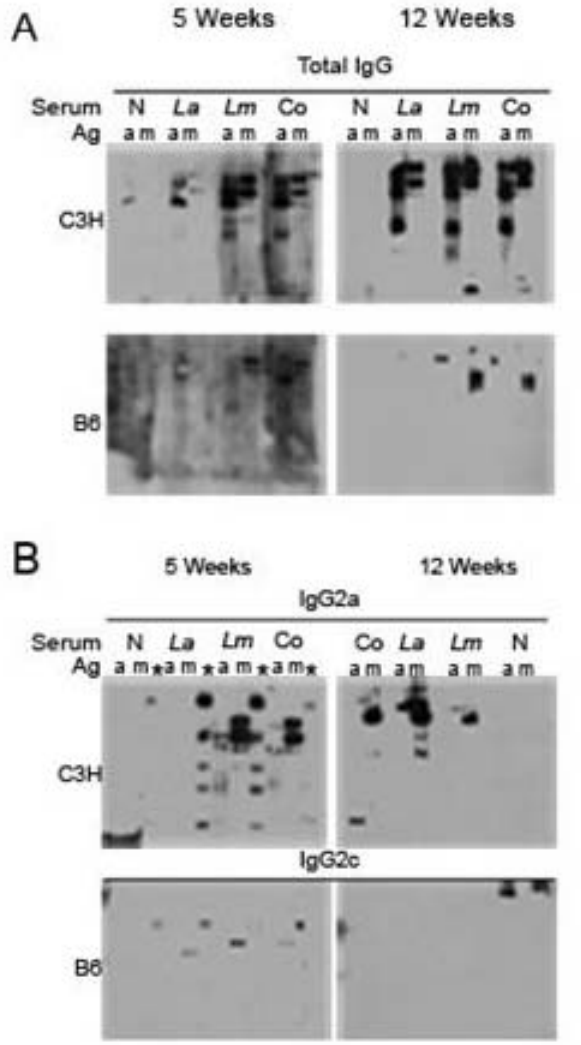
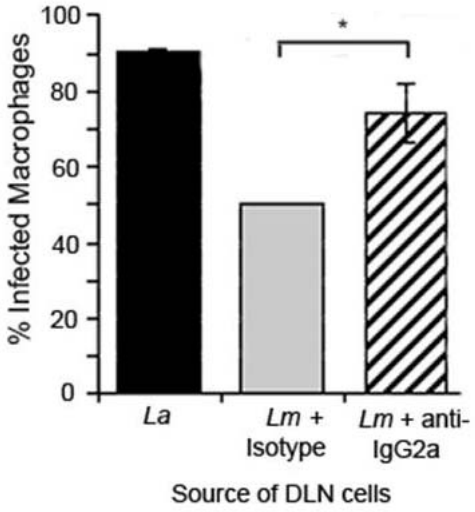


Figure 3. Western blot analysis of parasite-specific production of total IgG and IgG isotypes IgG2a and IgG2c. Freeze-thawed *Leishmania amazonensis* (a) or *Leishmania major* (m) antigen was separated on a polyacrylamide gel and protein was transferred to a polyvinylidene fluoride (PVDF) membrane. The blots were subsequently hybridized with mouse serum (1:25 dilution) pooled from four C3H mice (upper panel) or B6 mice (lower panel) that were non-infected (N) or infected for 5 or 12 weeks with *Lm*, *La*, or both parasites (Co), as indicated. Following serum hybridization, the membranes were probed with goat anti-mouse antibodies to **(A)** total IgG or with goat anti-mouse antibodies to **(B)** IgG2a (C3H, upper panels) or IgG2c (lower panels). * identifies lanes with commercial molecular weight markers that cross-reacted with serum antibodies. Results are from one experiment at 5 weeks p.i. and representative of two experiments at 12 weeks p.i.



Supplemental Figure. **IgG2a antibodies are necessary in vitro to kill *La* in infected macrophages.** Draining lymph node (DLN) cells from C3H mice infected with either *La* or *Lm* for 4 weeks were added to bone marrow-derived macrophages from C3H mice infected with *La* amastigotes on coverslips with *Lm* freeze-thawed antigen and incubated for 5 days at 34°C. Polystyrene beads were incubated with 12.5 µg/ml of either goat anti-mouse IgG2a or isotype control antibody, followed by 2 washing steps with PBS and a final concentration of 15 x 10^6 beads were added per well, as designated. * represent statistically significant differences ($p < 0.05$) as determined by unpaired student's t-test. Results are from 2 separate experiments.

Chapter 3

CO-INFECTION WITH *LEISHMANIA MAJOR* AND *LEISHMANIA AMAZONENSIS* PROMOTES A FUNCTIONAL B CELL GERMINAL CENTER RESPONSE IN C3HeB/FeJ MICE BUT NOT C57Bl/6 MICE

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Abstract

Co-infection of C3HeB/FeJ mice with both *Leishmania major* and *Leishmania amazonensis* leads to a healed footpad lesion, while co-infection of C57Bl/6 mice leads to chronic, non-healing lesions. This lack of healing corresponds to a B cell deficiency in stimulation of macrophage-mediated killing of *L. amazonensis* in vitro. Despite this knowledge, the mechanism behind the inability of C57Bl/6 mice to heal *L. amazonensis* is not known. Here we describe for the first time a difference in the draining lymph node germinal center B cell response between co-infected C3H and B6 mice. There are more germinal center B cells, more antibody isotype-switched germinal center B cells, more memory B cells and more antigen-specific antibody-producing cells in co-infected C3H mice compared to B6 mice as early as 2 weeks post-infection. We also show that IL-21 production in both mouse strains is similar at 2 weeks, suggesting the difference in these mouse strains is due to intrinsic B cell differences, rather than a difference in IL-21 production within germinal centers.

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Introduction

Leishmaniasis is a vector-borne disease caused by an obligate intracellular protozoan parasite of the genus *Leishmania*. Both *Leishmania (L.) major* and *L. amazonensis* cause cutaneous leishmaniasis, characterized by focal to multifocal cutaneous ulcerations which occur following infection by a sand fly bite. Infection of C3HeB/FeJ (C3H) mice with *L. major* results in self-cure within 8-12 weeks, dependent upon development of a polarized $CD4^+$T helper 1 (Th1) immune response. A Th1 response is critical for activation of macrophages to kill internalized parasites. Infection of the same mouse model with *L. amazonensis* leads to large, non-healing lesions and the immune response is not polarized to either a Th1 or Th2 response (2, 18), which is thought to lead to the lack of healing and disease progression (20, 23).

Prior infection of C3H mice with *L. major* leads to protection against subsequent *L. amazonensis* infection (26, 27). More recently it has been described that co-infection with both *L. major* and *L. amazonensis* in the same footpad leads to a healed lesion in C3H mice (7, 8). Compared to C3H mice, co-infected B6 mice have larger lesion sizes and a significantly higher lesion parasite load (7). Using an in vitro model of *Leishmania* infection developed in our laboratory we have identified that both $CD4^+$ T cells and $CD19^+$ B cells from *L. major*-infected C3H mice were necessary to kill *L. amazonensis* within infected macrophages (16). When $CD4^+$ T cells and $CD19^+$ B cells from B6 mice were utilized in this assay, killing was not observed (7). Using cell depletion studies it was determined that B cells from *L. major*-infected B6 mice do not have the same ability to activate infected

macrophages to kill *L. amazonensis* as B cells from C3H mice in this in vitro assay (7). It was also demonstrated that co-infected B6 mice are unable to make high levels of *Leishmania*-antigen specific antibodies as compared to co-infected C3H mice (7). In combination, these findings indicate the key role of B cells T-cell-mediated immune responses against *L. amazonensis*.

In this study we characterized the B cell response in both C3H and B6 mice co-infected with *L. major* and *L. amazonensis*. Here we describe novel differences in C3H and B6 B cell responses during *Leishmania* co-infection, specifically, increased germinal center B cell numbers, increased numbers of germinal center B cells that are isotype switched, increased memory B cells and increased antigen-specific antibody-producing cells in C3H mice as early as 2 weeks post-infection as compared to B6 mice. We also demonstrate that IL-21 production is not responsible for theses differences in B cell function, as both strains of mice have similar levels of IL-21-producing cells post-infection. Taken together our findings provide evidence that an adequate germinal center response is required to control a co-infection with *L. major* and *L. amazonensis*.

Materials and Methods

Mice. Female C57BL/6 (B6) and female C3HeB/FeJ (C3H) mice (6-8 weeks of age) were either obtained from Jackson Laboratories (Bar Harbor, Maine) or from an in-house breeding colony. Mice were maintained in a specific pathogen-free facility. Mice were infected with either 5 x 10^6 stationary-phase *L. major*, *L. amazonensis* or 2.5 x 10^6 *L. major* and 2.5 x 10^6 *L. amazonensis* promastigotes in

50µl of PBS in the left hind footpad. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Iowa State University.

Parasites and antigens. *L.amazonensis* (MHOM/BR/00/LTB0016) and *L. major* (MHOM/IL/80/Friedlin) promastigotes were grown in complete Grace's Insect medium (Atlanta Biologicals, Lawrenceville, GA) to stationary phase, harvested, washed in endotoxin-free PBS (Cellgro, Herdon, VA) and prepared to a concentration of 1 x 10^8 parasites per milliliter. Freeze-thawed *Leishmania* antigen (Ag) was obtained from stationary-phase promastigotes as previously described (10).

Lymph node cell culture and sorting. Total lymph node (TLN) cells were obtained from the left popliteal lymph node draining the site of infection from C3H and B6 mice infected for 2 or 5 weeks with *L. major, L. amazonensis,* or co-infected with both species. Lymph nodes from each mouse were kept separate and harvested into 2 ml of complete tissue culture medium (CTCM; RPMI 1640, 2mM L-glutamine, 100 U penicillin, 100µg streptomycin/ml, 25 mM HEPES, 0.05 µm 2-mercaptoethanol and 10% FBS). A single cell suspension was created using a 2 ml tissue homogenizer. Cells were passed through a 40µm nylon cell strainer (BD Falcon, Bedford, MA) and washed with 10 ml of CTCM at 250 x g, 4°C for 10 minutes. Following washing, cells were resuspended in 0.5 ml CTCM and counted.

Flow cytometry. For analysis of surface molecule expression, 0.5 x 10^6 total TLN cells were washed in 2 ml of fluorescence-activated cell sorting buffer (FACS, 0.1% sodium azide and 0.1% bovine serum albumin in phosphate buffer saline). Fcγ receptors were blocked with 10% purified rat anti-mouse CD16/CD32 antibody

(BD pharmigen, San Diego, CA) in 1mg/ml rat IgG (Sigma, St. Louis, MO) for 20 minutes at 4°C to prevent nonspecific binding. TLN cells were then incubated with appropriate primary antibody or isotype control for 30 minutes on ice. The antibodies used include phycoerythrin-labeled CD19(1D3), Cy5-labeled CD19 (1D3), biotin-labeled CD69, biotin-labeled IgM (b7-6), biotin-labeled IgD (11-26), biotin-labeled MHC class II (M5114 for B6 or I-A^k for C3H), fluorescein isothiocyanate-labeled CD86 (GL1), biotin-labeled CD40, fluorescein isothiocyanate-labeled PNA, phycoerythrin-labeled CD23 (B2B4), Cy5-labeled B220 (6B2) and biotin-labeled CD95. CD69, CD95 and MHC class II (I-A^k) were purchased from eBiosciences (San Diego, CA), PE-CD19 was purchased from BD pharmigen (San Diego, CA) and the remainder of antibodies were a gift and used as previously described (22). Following incubation, cells were washed twice in 2 ml of FACS buffer and then incubated with appropriate secondary antibody, if necessary, for 30 minutes at 4°C. Secondary antibodies included phycoerythrin-labeled streptavadin (as previously described (22)) and fluorescein isothiocyanate-labeled streptavadin (BD pharmigen (San Diego, CA)). After secondary antibody incubation, cells were washed twice in 2 ml FACS buffer, fixed in 200µl of 1% paraformaldehyde and stored at 4°C in the dark until analysis. Analysis was performed on a BD FACScanto flow cytometer (Becton Dickinson, San Jose, CA) and data analysis was performed using FlowJo software V8.5.2 (Tree Star, Inc., Ashland, OR).

Antigen-specific ELIspots. IgG1, IgG2a and IgG2c ELIspots were performed on TLN cells. Immulon 2 plates (Fischer, Fair Lawn, NJ) were coated with 5µg/ml of freeze-thawed *Leishmania* parasite antigen overnight at 4°C. Following washing

with PBS, commercially available biotinylated anti-IgG1, IgG2a and IgG2c antibodies (Jackson ImmunoResearch West Grove, PA) were added at a 1:10,000 dilution in 5% fetal bovine serum overnight at 4°C. ELIspots were developed using 2-amino-2-methyl-1-propanol (ICN Biomedicals Inc., Aurora, OH) and 5-bromo-4-chloro-3-indoly-phosphate (Fisher, Fair Lawn, NJ).

IL-21 ELIspots. A similar procedure was utilized as described above on TLN cells using the mouse IL-21 DuoSet kit according to manufacturer's instructions (R&D Systems, Minneapolis, MN.

Lymph node histopathology and immunohistochemistry. Popliteal lymph nodes from the left hind leg draining the site of infection were harvested and placed in cassettes in 10% neutral buffered formalin for histological and immunohistochemical analyses. Histologic examination was performed on paraffin-embedded tissues cut at 5 µm thickness onto positively charged slides and stained with Hematoxylin and Eosin (H&E). For immunohistochemistry, slides were deparaffinized and blocked with 20% normal rat serum. The sections were then incubated with an anti-mouse B220/CD45R antibody (BD Harlingen, San Diego, CA) overnight at a concentration of 1:400 with 10% normal rat serum. The slides were rinsed with PBS and then incubated with biotin-labeled anti-rat IgG (KPL, Gaithersburg, MD) at a concentration of 1:300 in 10% normal rat serum. Slides were washed and incubated with peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA) for 45 minutes. After 2 PBS washes, the color was developed with Nova Red (KPL, Gaithersburg, MD). The slides were then counterstained with Harris' hematoxylin, dehydrated and mounted with coverslips. Lymph nodes were

also frozen for immunohistochemical staining using peanut agglutinin (PNA). Frozen sections (OCT) were cut at 5 μm and fixed in cold acetone. Sections were washed once with PBS, blocked for 30 minutes with 10% normal rat serum then stained for 60 minutes with biotin-labeled PNA (Vector Laboratories, Burlingame, CA). Slides were then incubated with peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA) for 45 minutes. After 2 PBS washes, the color was developed with Nova Red. A semi-quantitative scoring scale for PNA staining was utilized as defined by: 0, no PNA staining; 1, 1-2 PNA-positive germinal centers, 2, 3-4 PNA-positive germinal centers; 3, 5 or more PNA-positive germinal centers; 4, greater than 6 PNA-positive germinal centers per lymph node. All evaluations were made based on the average of one lymph node section from 3 animals and two separate experiments.

Statistical Procedure. Statistical analysis was performed with Prism4 (GraphPad Software Inc., La Jolla, CA). Differences between groups were determined using a Mann-Whitney T-test. P-values < 0.05 were considered statistically significant.

Results

There are fewer germinal center B cells and isotype switched germinal center B cells during co-infection of B6 mice than C3H mice.

We previously demonstrated that *L. major* and *L. amazonensis* co-infection of C3H mice heal footpad lesions by 10-12 weeks post-infection. Co-infected B6 mice, in comparison, have persistent non-healing lesions and a higher footpad parasite

burden (7). Using an in vitro co-culture assay, we have shown that B cells harvested from *L. major*-infected B6 mice do not function to kill *L. amazonensis* in contrast to B cells from *L. major*-infected C3H mice (7). Based on these previous findings, we hypothesized that B cells from co-infected B6 would be phenotypically and functionally different from B cells from co-infected C3H mice. Using draining TLN cells from mice infected with *L. amazonensis*, *L. major* or co-infected with both species for 2 and 5 weeks, we assessed the number of germinal center B cells and isotype-switched germinal center B cells via a 3-color flow cytometric analysis.

Upon entering the germinal center, B cells typically display the peanut agglutinin (PNA) lectin and upregulate CD95 surface expression (17). There were significantly more germinal center positive ($B220^+$, PNA^+) B cells in the draining lymph nodes of co-infected C3H mice as compared to co-infected B6 mice at both 2 and 5 weeks post-infection (Figure 1A). Naïve mice of both strains had negligible numbers of germinal center B cells (Figure 1A).

The germinal center functions as the primary location for isotype switching of activated B cells. Cells that are PNA^+ and IgM^- have been shown to be germinal center B cells that have undergone isotype switching (5). To assess the population of B cells within this phenotype we assessed the $B220^+$, PNA^+ cell populations via FACS analysis of cells from the draining lymph nodes of *L. amazonensis*, *L. major* and co-infected C3H and B6 mice for expression of IgM. We determined that co-infected C3H mice have more germinal center B cells that are IgM^- and therefore have undergone isotype switching at 2 and 5 weeks post-infection (Figure 1B). Upon activation, B cells will also up-regulate surface expression of MHC class II,

CD80, CD86 and CD40 (13). To assess B cell activation status, we also used flow cytometry to determine surface expression of these markers. Draining lymph node cells were fist gated on the $B220^+$ population and no differences were observed in the mean fluorescent intensity (MFI) over naïve of MHC class II (Figure 1C) and CD86 (Figure 1D) on B cells during a infection with a single species of the parasite or during the co-infection at either 2 or 5 weeks. Similarly, no differences in CD40 expression was noted during any of the infection groups at either time point (data not shown). These findings demonstrate that there is no difference in the B cell activation status of co-infected B6 and C3H mice following *Leishmania* infection.

To confirm our flow cytometric findings we performed immunohistochemistry using anti-B220 (also known as CD45R) and biotin-labeled PNA on draining lymph nodes of co-infected mice 2 week post-infection. The pattern of immunoreactivity for B220 demonstrated that lymph nodes from co-infected C3H mice have active cortices with multiple, large follicles and distinct germinal center formation, as compared to co-infected B6 mice which had less distinct follicles and rare germinal centers (Figure 2A, top panels). PNA staining confirmed there were more germinal centers in co-infected C3H mice, and draining lymph nodes of co-infected B6 mice had germinal centers that were smaller and fewer in number (Figure 2A, bottom panels and B). Together, these findings indicate that the germinal center B cell response at both 2 and 5 weeks post-infection in co-infected B6 mice was less robust as characterized by fewer germinal center B cells and fewer germinal center B cells that had undergone isotype switching. It is known that the germinal center is the site in which B cells either become memory B cells or antibody-secreting plasma

cells (3), therefore these findings may suggest that C3H mice that are co-infected with *L. major* and *L. amazonensis* may more memory B cells and/or more antibody-secreting cells.

During co-infection, there are more memory B cells in C3H mice as compared to B6 mice.

Based on finding more germinal center B cells in co-infected C3H mice than B6 mice, we wanted to determine if this difference in the number of germinal center B cells would lead to a downstream difference in the memory B cell population between these two mouse strains after co-infection. TLN cells were analyzed via flow cytometry with anti-CD19 to identify B cells, anti-IgM and anti-CD23. Anti-CD19, instead of anti-B220, was used to eliminate plasmacytoid dendritic cells which also express B220. Non-switched, immature B cells have previously been shown to express surface IgM and CD23, while memory B cells are IgM^-, $CD23^-$ (22). At both 2 and 5 weeks after co-infection we found that C3H mice had increased numbers of memory B cells as compared to B6 mice (Figure 3). During single infection with either *L. major* or *L. amazonensis*, C3H mice also had a larger population of memory B cells than B6 mice (Figure 3). We showed in figures 1 and 2 that germinal center formation was augmented in C3H but not B6 mice after co-infection. Given the critical role for germinal center formation in creation of effector B cells, it makes sense that both isotype switching and creation of memory B cells is compromised if germinal center formation is lackluster, as seen after co-infection of B6 mice.

Co-infected C3H mice produce more antigen-specific antibodies than co-infected B6 mice.

In order to determine if the observed differences in germinal center formation and B cell effector phenotype lead to differences in B cell antibody production, we analyzed the number of antigen-specific antibody-producing B cells during co-infection of C3H and B6 mice. ELIspot analysis was performed on draining TLN cells for antigen-specific antibody production of IgG2a (C3H), IgG2c (B6) and IgG1 from draining lymph nodes of co-infected C3H and B6 mice. B6 mice have previously been shown to carry the Igh1-b allele that encodes for and leads to IgG2c antibody isotype production, while C3H mice carry the Igh1-a allele and therefore produce IgG2a (14), which is why we measured production of these two specific antibody isotypes. C3H mice produce more *L. major*-specific IgG2a than B6 mice produce *L. major*-specific IgG2c at both 2 and 5 weeks post-infection (Figure 4A). Differential production of *L. major*-specific antibody is associated with a healing response against *L. amazonensis* during the co-infection (7). No significant differences were noted in the production of antigen-specific IgG1 (Figure 4B). These findings indicate there is a differential germinal center B cell response in co-infected C3H mice, leading to the production of more antigen-specific antibodies as compared to B6 mice.

IL-21 production is similar between C3H and B6 mice co-infected for 2 weeks

When B6 mice are co-infected in the footpad with both *L. major* and *L. amazonensis* they have fewer germinal centers within the draining lymph node

which contain fewer B cells, fewer isotype switched germinal center B cells, fewer memory B cells and fewer antigen-specific IgG2c antibody-producing cells than co-infected C3H mice. We sought to determine if these differences have to do with a differential production of interleukin (IL)-21 from the draining lymph node of co-infected C3H versus B6 mice. IL-21, produced by T follicular helper (Tfh) cells within germinal centers, has been shown to function in B cell proliferation and production of plasma cells (9). B cells express the receptor for IL-21, and B cells deficient in this receptor have an impaired ability to undergo isotype switching and cannot maintain germinal center organization (6). We performed IL-21 ELIspots on TLN cells from co-infected C3H versus B6 mice and found that there was no difference in the number of IL-21 producing cells at 2 weeks post-infection with or without antigen stimulation (Figure 5 and data not shown). A paucity of IL-21 production is unlikely to be responsible for the differences we have described in germinal center B cell responses after co-infection of B6 mice versus C3H mice.

Discussion

It has yet to be determined what immune factors are required to heal cutaneous Leishmaniasis caused by *L. amazonensis*. Previous work has described that footpad co-infection with both *L. major* and *L. amazonensis* leads to a healing phenotype in C3H mice, while B6 mice develop non-healing, persistent lesions (7, 8). We have also determined that, using an in vitro model that mimics the co-infection, B cells from infected B6 mice do not function as effectively as B cells from C3H mice to kill intracellular L*. amazonensis* (7). Here we describe for the first time a

difference in the germinal center B cell response between C3H and B6 mice co-infected with *L. major* and *L. amazonensis* early in infection. Germinal centers, formed within secondary lymphoid organs, are the site for early B cell expansion (24). Following proliferation, signal-dependent isotype switching occurs within the germinal center, changing the B cell receptor surface expression from IgD or IgM to IgG, IgA or IgE (24). At 2 and 5 weeks following infection there were more germinal center B cells and more isotype switched germinal center B cells in co-infected C3H mice compared to B6 mice (Figure 1A and B). We also demonstrated that there were increased germinal centers within the draining lymph nodes of co-infected C3H mice compared to co-infected B6 mice at 2 weeks post-infection (Figure 2A and B). B cell memory is also formed in germinal centers (3). Given this additional role for germinal centers, it was not surprising that there are more memory B cells at both 2 and 5 weeks post co-infection in C3H mice compared to B6 mice (Figure 3). Immunological memory is required for an accelerated and robust immune response to pathogens (11), and this finding suggests that without a good memory B cell response co-infection with *L. major* and *L. amazonensis* cannot be controlled, as indicated by large, non-healing lesions in co-infected B6 mice.

The germinal center is an essential site in which there is generation of memory B cells and isotype switching which function to produce effector B cells and antigen-specific antibody-producing cells (22). Little has been documented regarding the germinal center response during *Leishmania* infection, as this pathogen is considered to classically activate a Th1 polarized immune response. Histological studies in mice have reported enlarged or hyperplastic germinal centers

with apoptotic cells up to 40 days post-infection with *L. amazonensis* (1). More recently using *L. major* infection of BALB/c mice it was determined that Tfh cells in the germinal centers produce cytokines that influence the affinity and isotype of the antibody response (19). Our data indicates that there are more antigen-specific IgG2a-producing cells in the draining lymph node of co-infected C3H mice as compared to antigen-specific IgG2c-producing cells from co-infected B6 mice (Figure 4). These two antibody isotypes are important, as they are the predominant antibodies produced during a polarized Th1 immune response (4). The presence of robust germinal center formation in C3H mice versus B6 mice after co-infection and the downstream effector function of B cells as measured by isotype switching, memory cell formation and production of antigen-specific antibodies all suggest a robust germinal center response is required for a productive immune response and healing of co-infection with *L. major* and *L. amazonensis*.

The role of B cells during infection with *Leishmania* is controversial. Some reports have described a protective role for both B cells and antibodies during *Leishmania* infection. Scott et. al showed that blocking B cell production of antibodies in neonatal mice using an anti-mu antibody impaired the T cell-mediated immune response following *L. major* infection (21). Antibody production has also been shown to be a key factor for phagocytosis of *L. major* by dendritic cells. Without antibody opsonization, infected mice had larger lesion sizes, higher parasite loads, lower interferon (IFN)-γ production and a decreased T cell response (29). Other studies describe a negative effect of antibody production during *Leishmania* infection. When IgG-negative BALB/c mice were infected with *L. major*, smaller

lesions with a lower parasite load compared to infected mice with IgG were observed (15). A more recent study showed *L. amazonensis*-infected mice that lacked functional B cells and therefore antibodies, had a delayed onset of disease and developed small lesions (28). It has also been shown that there were limited infections with both *L*. *amazonensis* and *L*. *pifanoi* in the absence of circulating antibodies, and infection of Fc gamma receptor (FcγR) knockout mice resulted in similarly limited lesions (12). When FcγRIII knockout mice were infected with *L. mexicana* lesions failed to develop. Instead, these mice produced high levels of IFN-γ, indicating there is a negative effect when antibodies bind FcγRIII during *L. mexicana* infection (25). Although there appears to be a discrepancy for the role of B cells during *Leishmania* infection, B cells appear to be playing an important role during a co-infection of C3H mice with *L. major* and *L. amazonensis*, while no positive role for B cells is indicated in co-infected B6 mice.

It is known that T cells have a critical role in germinal center formation and maintenance. T follicular helper (Tfh) cells are a T helper cell subset that is specialized in regulating the effector and memory responses of B cells (6). Within the germinal center Tfh cells produce the cytokine IL-21 which has been shown to promote B cell proliferation and production of plasma cells (9). B cells express the IL-21 receptor and B cells deficient in this receptor have an impaired ability to undergo isotype switching and lose germinal center organization (6). We proposed that differential IL-21 production might be responsible for the germinal center phenotypic differences we determined between co-infected C3H and B6 mice. Despite this, there was no difference in the number of IL-21-producing cells at 2

weeks post-infection between these two mouse strains following antigenic stimulation (Figure 5). This determination suggests that Tfh helper cells in both mouse strains produce similar amounts of IL-21 so the defects in germinal center formation and function in co-infected B6 mice may be due to disparate B cell responsiveness, not a Tfh cell defect. Future studies would look at expression of the IL-21 receptor on B6 B cells as compared to C3H B cells during the co-infection, to determine if there is differential expression of this receptor which would explain the differences we see in germinal center formation and effector B cell responses.

Acknowledgements

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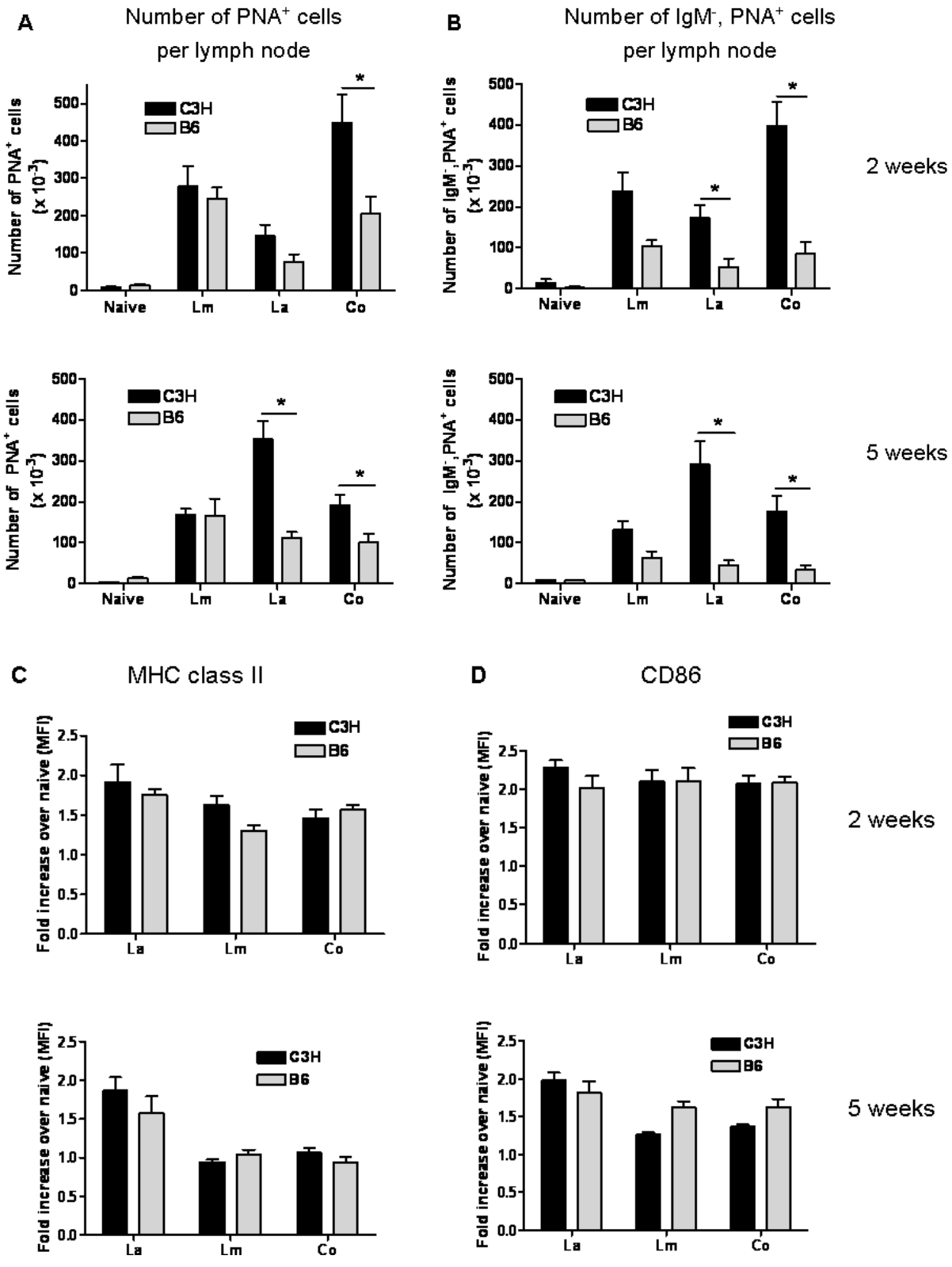
A
Number of PNA+ cells per lymph node
Number of PNA+ cells (x 10-3)
C3H
B6
Naive
Lm
La
Co
2 weeks
5 weeks
B
Number of IgM-,PNA+ cells per lymph node
Number of IgM-,PNA+ cells (x 10-3)
C
MHC class II
Fold increase over naive (MFI)
D
CD86

Figure 1. Increased number of both germinal center B cells and germinal center B cells undergoing isotype switching in co-infected C3H mice. C3H (black) and B6 (gray) mice were infected with *L. amazonensis*, *L. major*, or co-infected with both species of parasites. Total draining lymph node cells were harvested at 2 and 5 weeks post-infection. Cells were first gated on the $B220^+$ population and analyzed for (**A**) binding to PNA (**B**) PNA binding and IgM surface expression (**C**) surface expression of MHC class II and (**D**) surface expression of CD86 as indicated by mean fluorescence intensity (MFI). Cell number was determined based on the percentage of cells within the gated population and the total number of lymph node cells recovered. MFI for each marker presented as fold increase over naïve control. Data are represented as the mean ± SEM of three separate experiments; *P ≤ 0.05. Lm, *L. major*; La, *L. amazonensis*; Co, co-infected with both *L. major* and *L. amazonensis*.

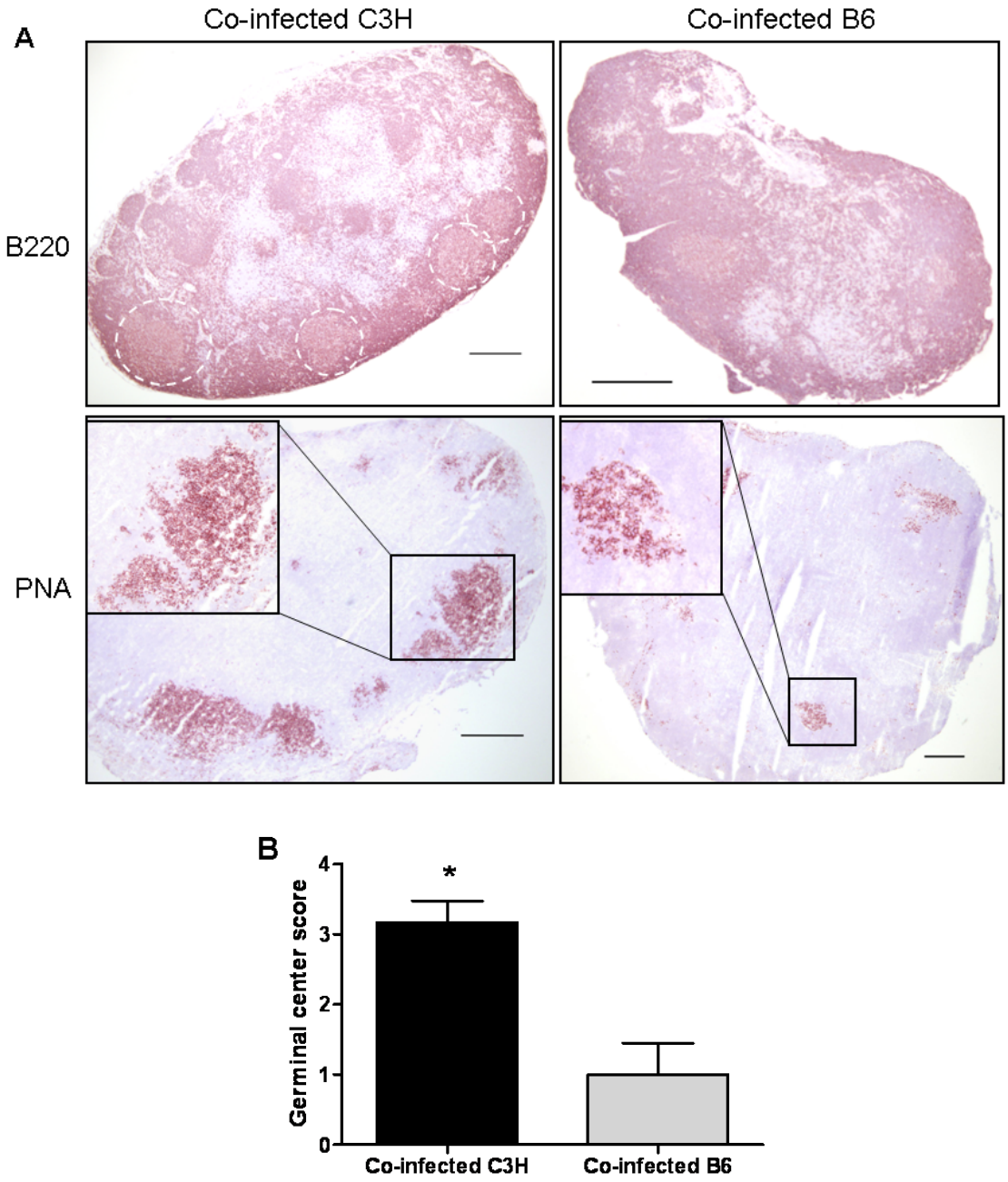

Figure 2. More germinal centers following co-infection of C3H mice with *L. major* and *L. amazonensis*. A. Photomicrographs of lymph node sections labeled with anti-mouse B220 (top panels) and biotin-PNA (bottom panels) from C3H and B6 mice co-infected for 2 weeks. White dash lines delineate germinal centers. Bar = 200μm. **B.** Histologic germinal center scores for PNA immunoreactivity at 2 weeks. Score is based on the number of PNA^+ germinal centers within a single draining lymph node. Data are representitive of 2 separate experiments ± SEM with 3-4 mice per group, per experiment. *P=0.0087.

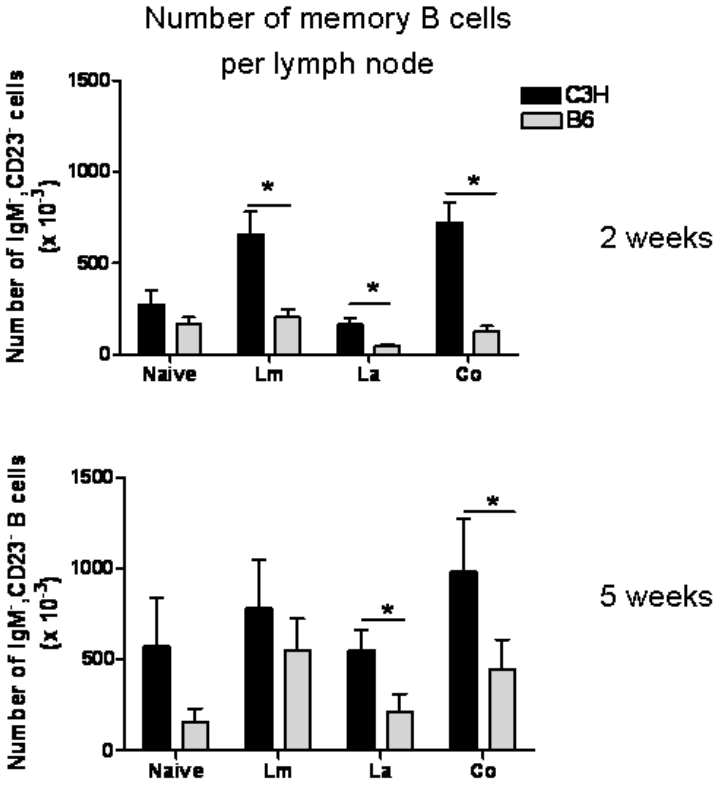

Figure 3. Increased number of memory B cells ($B220^+$,IgM^-,$CD23^-$) in the draining lymph node of co-infected C3H mice. C3H (black) and B6 (gray) mice were infected with *L. amazonensis*, *L. major*, or co-infected with both species of parasites. Total draining lymph node cells were harvested at 2 and 5 weeks post-infection. Via flow cytometry, cells were gated on a $CD19^+$ population and surface expression of both IgM and CD23 was determined based on the percentage of cells within the gated population as compared to the total lymph node cells recovered. Data are representitive of two separate experiments ± SEM; *$P \leq 0.05$. Lm, *L. major*; La, *L. amazonensis*; Co, co-infected with both *L. major* and *L. amazonensis*.

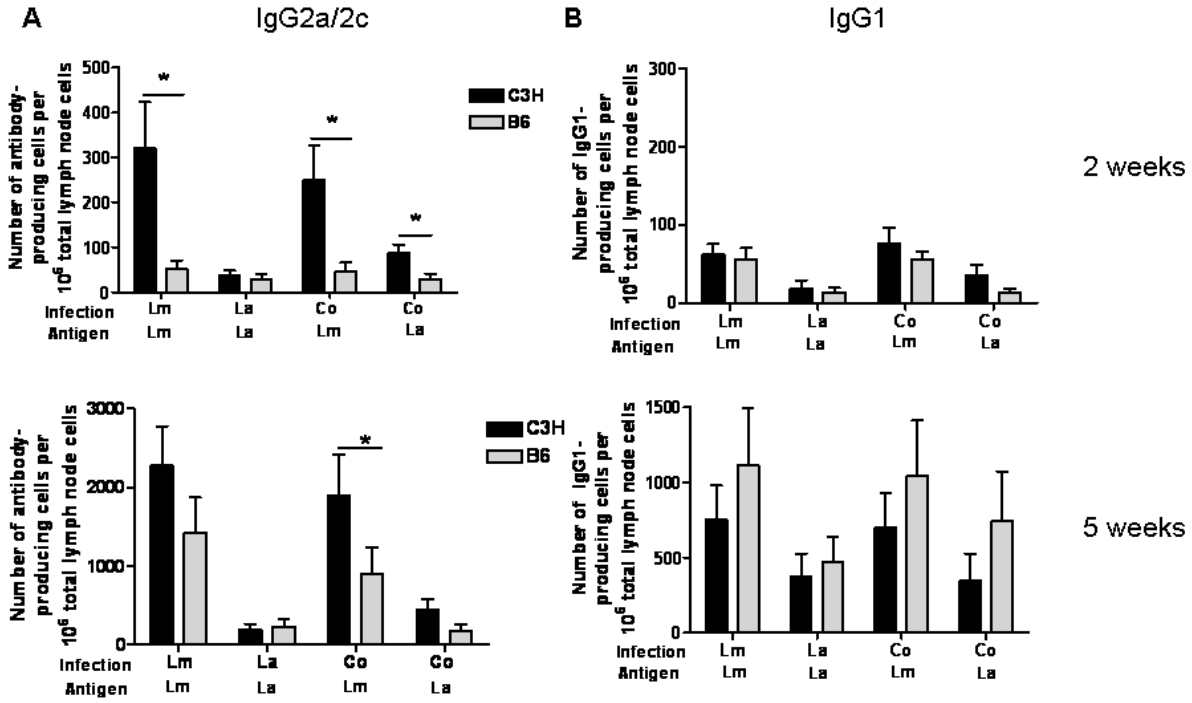

Figure 4. Increased number of antigen-specific IgG2a-producing cells following co-infection in C3H mice as compared to antigen-specific IgG2c-producing cells in co-infected B6 mice. C3H (black) and B6 (gray) mice were infected with *L. amazonensis*, *L. major*, or co-infected with both species of parasites. Total draining lymph node cells were harvested at 2 and 5 weeks post-infection (please note scales). Number of (**A**) IgG2a (C3H) and IgG2c (B6) producing cells and (**B**) IgG1-producing cells as determined by ELIspot analysis of total draining lymph node cells stimulated with freeze-thawed *Leishmania* promastigote antigen, as indicated above. Data are represented as the mean ± SEM of three separate experiments; *P ≤ 0.05. Lm, *L. major*; La, *L. amazonensis*; Co, co-infected with both *L. major* and *L. amazonensis*.

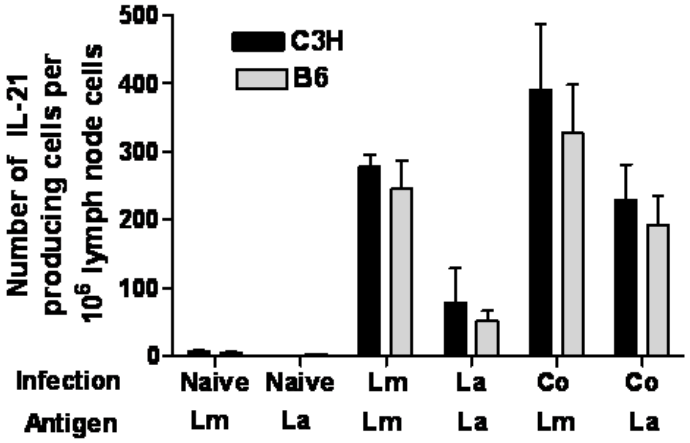

Figure 5. No difference in the number of IL-21 producing cells in draining lymph node cells from co-infected C3H and B6 mice. Number of IL-21-producing cells was determined by ELIspot analysis of total draining lymph node cells stimulated with freeze-thawed *Leishmania* promastigote antigen. Data are represented as the mean ± SEM of two (for B6) or three (for C3H) separate experiments. Lm, *L. major*; La, *L. amazonensis*; Co, co-infected with both *L. major* and *L. amazonensis*.

Chapter 4

THE FcGAMMA RECEPTOR-NADPH OXIDASE PATHWAY AND ANTIBODY-ENHANCED INTRACELLULAR KILLING OF*LEISHMANIA AMAZONENSIS*

A manuscript prepared for submission to the Journal of Immunology

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Abstract

Leishmania (L.) amazonensis is a causative agent of cutaneous leishmaniasis in humans and can lead to severe, disseminated disease. Mice infected with *L. amazonensis* have a non-polarized T helper cell response and non-healing, chronic lesions. In vitro, a productive response to this pathogen has been recapitulated through macrophage production of both nitric oxide and superoxide. We show FcγR and cytochrome b558 are necessary for superoxide production during an established infection. We demonstrate NADPH oxidase assembly of $gp91^{phox}$ and $p67^{phox}$ occurs by day 1 during the in vitro infection and is localized directly adjacent to the parasite. However, measurable superoxide production was only detectable at day 5 in vitro, indicating that assembly of these subunits was not sufficient to trigger superoxide production. Using wortmannin inhibition of PI3K, we show inhibition of superoxide production at day 5 and indicating that PI3K is critical for superoxide production at this late stage of infection. These data establish that the FcγR-NADPH oxidase activation pathway is required to kill intracellular *L. amazonensis*.

We propose that this novel pathway requires *L. major* antigen-specific B cell

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production of antibodies which bind stimulatory Fcγ receptors to produce superoxide through PI3K-mediated activation of assembled NADPH oxidase complexes that are associated with intracellular amastigote parasites. Understanding the role of this pathway in controlling non-healing cutaneous leishmaniasis caused by *L. amazonensis* may be critical in determining specific immunomodulation to successfully treat this disease.

Introduction

Leishmaniasis is a vector-borne zoonotic disease caused by protozoa parasites of the genus *Leishmania*. *Leishmania (L.) major* and *L. amazonensis* both cause cutaneous leishmaniasis which is manifested as focal to multifocal skin lesions (28). Lesions caused by *L. major* are often self-curing while *L. amazonensis* can lead to chronic, disseminated disease (1, 28). Parasite killing within infected macrophages occurs when there is initiation of an appropriate immune response which sufficiently activates infected macrophages. *L. major* has been shown to initiate a strong polarized T helper 1 (Th1) immune response characterized by a $CD4^+$ T cell population that produces of interferon gamma (IFN-γ) to classically activate macrophages (28). In contrast *L. amazonensis* does not produce a polarized Th1 immune response. *L. amazonensis* antigen-specific $CD4^+$ T cells have been shown to allow both disease progression and pathology (1, 27).

Macrophages are classically activated by IFN-γ and exposure to microbes or microbial-derived products (17, 21). These macrophages have an increased ability to degrade and destroy intracellular organisms due to phagolysosomal production of

reactive oxygen species and nitric oxide (NO) (21). Inducible nitric oxide synthase (iNOS) has been shown to catalyze formation of reactive nitrogen intermediates including NO and/or other reactive nitrogen molecules (17). Multiple studies suggest that virulence and chronicity of *L. amazonensis* infection in mouse models may be related to parasite resistance of NO-mediated killing (10, 23, 29). Recently, several different human clinical isolates of *L. amazonensis* and *L. braziliensis* were demonstrated to positively correlate lesion severity and parasite resistance to nitric oxide (9). NO may only be cytostatic for *L. amazonensis* versus cytotoxic as it is for *L. major*. Cytotoxicity activity against *L. amazonensis* was shown to be dependent on formation of peroxynitrite and/or compounds derived from peroxynitrite (16).

Reactive nitrogen species may interact with NADPH-oxidase-dependent superoxide to produce or enhance the cytotoxic response (reviewed in (3)). The primary mechanism of superoxide generation has been shown to occur during phagocytosis through assembly of NADPH oxidase complexes on the phagosomal cup and membrane (24). The NADPH complex is composed of two membrane-bound subunits, $gp91^{phox}$ and $p22^{phox}$, which form cytochrome b558 (cytoB), four cytosolic subunits, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small GTPase Rac (24). Assembly of NADPH oxidase is triggered via binding of complement receptors and/or Fcγ receptors (FcγR) on the membrane of the macrophage to complement components or the Fc domain of antibodies, respectively (25), (12). FcγR signaling promotes association of NADPH oxidase cytosolic components with cytoB and activation of the enzyme produces superoxide. A recent study demonstrated that assembly of NADPH oxidase complexes can occur without superoxide production,

and production of superoxide requires both the $p40^{phox}$ subunit and PI3K activation in addition to complex assembly (31).

We have previously demonstrated that macrophage killing of *L. amazonensis* requires both NO and superoxide production (23). We have also determined both $CD4^+$ T cells and B cells are important factors for resolution of *L. amazonensis* infection (22). From the results shown here we suggest the role of $CD4^+$ T cells is to activate iNOS production of NO via production of IFN-γ, which binds to the IFN-γ receptor (IFN-γR). We also suggest that the role of B cells is to produce antibodies that bind stimulatory Fc gamma receptors (FcγR) to activate NADPH oxidase production of superoxide via the PI3K signaling pathway and they can be effective after the establishment of the intracellular infection. We demonstrate that NADPH oxidase subunit assembly of $gp91^{phox}$ and $p67^{phox}$ occurs adjacent to the parasite and as early as day 1 during in vitro co-culture. Although we observe assembly of these subunits early, measurable superoxide is not produced until day 5 which corresponds with when we observe intracellular killing of *L. amazonensis*. When the PI3K inhibitor wortmannin is added to *L. amazonensis*-infected macrophages in co-culture, we do not see production of superoxide, indicating that PI3K signaling is critical for production of superoxide in this system. The requirements for both FcγR and cytochrome b558 signals to produce formazan precipitants indicative of superoxide production along with a PI3K-dependent signal is a novel mechanism to reduce an established intracellular parasitic infection.

Materials and Methods

Mice and infection. Female C57BL/6 (B6) mice, IFN-γ receptor (IFN-γR$^{-/-}$), inducible nitric oxide synthase (iNOS$^{-/-}$) and Cytochrome b558 (CytoB$^{-/-}$) deficient mice (all on B6 background) (6-8 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, Maine). Fcγ receptor common chain deficient (FcγR$^{-/-}$) mice on a B6 background were kindly donated by Dr. Mary Ann McDowell at the University of Notre Dame. Mice were maintained in a specific pathogen-free facility. Female C3HeB/FeJ (C3H) (6-8 weeks of age) were obtained from an in-house breeding colony. C3H mice were infected with 5 x 10^6 stationary-phase promastigotes in 50µl of PBS in the left hind footpad. For the propagation of lesion-derived amastigotes female C3H severe combined immunodeficient (SCID) mice were infected with 20 x10^6 stationary-phase promastigotes in 50µl of PBS in the left hind footpad. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Iowa State University.

Parasites and antigens. *L.amazonensis* (MHOM/BR/00/LTB0016) and *L. major* (MHOM/IL/80/Friedlin) promastigotes were grown in complete Grace's Insect medium (Atlanta Biologicals, Lawrenceville, GA) to stationary phase, harvested, washed in endotoxin-free PBS (Cellgro, Herdon, VA) and prepared to a concentration of 1 x 10^8 parasites per milliliter. Freeze-thawed *Leishmania* antigen (Ag) was obtained from stationary-phase promastigotes as described (13).

Cells and cell culture. Bone marrow cells were harvested from femurs and tibias of wild-type B6 or previously mentioned null mice and plated in 150 x 15 mm Petri dishes with 30ml of macrophage medium (30% L-cell conditioned medium, 20% fetal

bovine serum (FBS), 50% Dulbecco's modification of eagle's medium (DMEM), 2mM L-glutamine, 100 U penicillin per ml, 100µg of streptomycin per ml and 1 mM sodium pyruvate) for 6 days at 37°C and 5% CO_2. On day 7 the adherent cell population was collected and washed with PBS. Trypan blue exclusion was used to determine the number of live cells followed by resuspension of cells in complete tissue culture medium (CTCM; DMEM, 2mM L-glutamine, 100 U penicillin, 100µg streptomycin/ml, 25 mM HEPES, 0.05 um 2-mercaptoethanol and 10% FBS).

Macrophage infection. *L.major* and *L. amazonensis* amastigotes were collected from infected footpads of SCID mice and were used to infect bone marrow-derived macrophages (BMM) at 3:1 parasite to cell ratio and incubated at 34°C with 5% CO_2 in 24 well plates seeded with tissue coverslips. After 24 hours, macrophages were washed two times in PBS to remove extracellular amastigotes.

Lymph node cell culture and sorting. Total lymph node (TLN) cells were obtained from the left popliteal lymph node draining the site of infection from C3H mice infected for 4 weeks with *L. major*. Lymph nodes from 10-15 mice were pooled into 2 ml of CTCM and a single cell suspension was created using a 2 ml tissue homogenizer. Cells were washed with 10 ml of CTCM at 250 x g, 4°C for 10 minutes. Following washing, cells were resuspended in 5 ml CTCM, passed through a 40µm nylon cell strainer (BD Falcon, Bedford, MA) and counted via trypan blue exclusion. $CD4^+$ T cells (Miltenyi Biotech, Auburn, CA) or $CD19^+$ B cells (MagCellect, R&D system, Minneapolis, MN) were purified via depletion using an autoMACS^{TM} separator (Miltenyi Biotech, Auburn, CA). TLN cells or purified $CD4^+$ T cells and B cells were added to the top compartment of 0.4 µm diameter transwells

with freeze-thawed *L. major* promastigote antigen. Following sorting, the cells were washed and resuspended in 1 ml CTCM and counted again. Where indicated, co-cultures were pre-incubated with 100nM wortmannin (Sigma, St. Louis, MO) at 37°C for the time indicated. Cell purity was assessed using FACS analysis.

Determination of macrophage infection rate. Following incubation for 1 to 5 days, coverslips were harvested, fixed and stained using nonspecific HEMA 3 stain set (Fisher Diagnostics, Middletown, VA). Coverslips were mounted onto glass slides and counted via light microscopy at 100x oil magnification. Three areas of 100 cells each were counted for parasite infection rate.

Determination of nitric oxide and superoxide production. The concentration of nitrite was assessed using Greiss reagent as described previously (18). Briefly, 50 µl of cell culture supernatant and 50 µl of Greiss reagent (LabChem, Pittsburgh, PA) were mixed and incubated at room temperature and the absorbance was measured at 550nm with a microplate reader (Molecular Devices, Sunnyvale, CA). Nitrite concentration was determined using a standard curve generated with sodium nitrite. Production of superoxide was assessed using Nitro Blue Tetrazolimide (NBT) (Sigma, St. Louis, MO) tablets. NBT tablets were dissolved in 1ml sterilized deionized water and 30 µl of NBT was added to designated wells and incubated for 60-90 minutes at 37°C, 5% CO_2. Coverslips were harvested, fixed and stained with eosin. Presence of formazan, indicative of superoxide production, was based on visualization of basophilic (blue) precipitants within cells using light microscopy as follows: 0, no formazan; 1, 1-5 cells with formazan; 2, 6-10 cells with formazan; 3, >

10 cells with formazan. All evaluations were calculated from the mean of 10, 40x fields per coverslip, 2 coverslips per experiment and 2-3 separate experiments.

Immunofluorescence. Following infection and co-culture for the designated number of days, coverslips were harvested and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature and washed three times with PBS. BMM were permeabilized with 0.01% Triton X in PBS for 10 minutes at room temperature. Cells were incubated overnight at 4°C with goat anti-mouse $gp91^{phox}$, rabbit anti-mouse $p67^{phox}$ (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa Fluor CD107A (LAMP1) (eBiosciences, La Jolla, CA) at a 1:50, 1:100 and 1:100 dilution in PBS, respectively. After incubation, coverslips were washed three times with PBS and incubated for 1 hour at room temperature with anti-goat Cy3-conjugated antibody or anti-rabbit Cy2-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:200 dilution in PBS, if necessary. BMM were then counterstained with 4'6-Diamidino-2-phenindole (DAPI) (Sigma, St. Louis, MO). Coverslips were mounted onto slides using MOWIOL (Calbiochem, La Jolla, CA) and viewed via sequential scanning confocal microscopy using an Olympus IX81 inverted microscope (Olympus America Inc., Center Valley, PA). Pearson's co-localization coefficient was determined via Olympus Fluoview version 2.1b software and a single coefficient was measured per one field of view.

Statistical Procedure. Statistical analysis was performed with Statview (SAS, Cary, NC) using ANOVA, Scheffe's post-hoc test and linear regression for figures 1 and 3 and via Prism4 (GraphPad Software Inc., La Jolla, CA) for figures 2, 6 and

Table 1. Differences between groups were determined using a Mann-Whitney T-test. P-values < 0.05 were considered statistically significant.

Results

L. amazonensis-infected macrophages require IFN-γ receptor, iNOS, Fcγ receptor common chain and the cytochrome B subunit of NADPH oxidase for killing of the parasite.

In our laboratory we have developed an in vitro killing assay in which bone marrow-derived macrophages (BMM) are infected with *L .amazonensis* amastigotes for 24 hours and then co-cultured with total lymph node (TLN) cells or purified $CD4^+$ T cells and B cells isolated from C3H mice infected with *L. major* (22). Five day co-culture with either TLN or purified cells from *L. major*-infected mice promotes a significant decrease in the percent *L. amazonensis*-infected macrophages (22). Based on these findings, along with previous knowledge that both nitric oxide and superoxide are necessary for macrophage activation to kill *L. amazonensis* (23), we proposed that interferon gamma receptors (IFN-γR) on macrophages would be necessary for activation of inducible nitric oxide synthase (iNOS) and induction of nitric oxide (NO) to kill intracellular parasites. We also hypothesized that stimulatory Fc gamma receptors (FcγR) that bind immunoglobulin (Ig)G antibodies and NADPH oxidase would be required for production of superoxide within the phagolysosome to kill *L. amazonensis*. To test these hypotheses we used BMM from wild-type C57Bl/6 (B6) mice along with BMM from IFN-$\gamma R^{-/-}$,$iNOS^{-/-}$, $Fc\gamma R^{-/-}$ and $CytoB^{-/-}$ mice on a B6 background. BMM were harvested, infected with *L. amazonensis* and co-cultured

with either TLN cells or purified $CD4^+$ T cells and B cells from the lymph nodes of mice infected for 4 weeks with *L. major*. A transwell system was utilized so cells from different mice were never in direct contact. *L. amazonensis*-infected BMM co-cultured with total or purified lymph node cells from *L. major*-infected mice were not able to kill intracellular parasites in the absence of IFN-γ, iNOS, FcγR or CytoB (Figure 1A, black bars). In contrast $Fc\gamma R^{-/-}$ or $CytoB^{-/-}$ BMM had non deficit in killing L. major, while $IFN\gamma\text{-}R^{-/-}$ and $iNOS^{-/-}$ BMM infected with *L. major* were not able to kill the parasite. Therefore, as previously described, killing of intracellular *L. major* requires the presence of IFN-γR and iNOS. In comparison, killing of *L. amazonensis* within macrophages requires the presence of IFN-γR, iNOS, FcγR and Cytochrome b558.

The presence or absence of superoxide was confirmed in wild-type and null macrophages after *L. amazonensis* infection via the nitro blue tetrazolium (NBT) assay, which detects production of superoxide via formation of a blue formazan precipitate. There was no superoxide production from $Fc\gamma R^{-/-}$ or Cyto $B^{-/-}$ macrophages infected with *L. amazonensis* in the co-culture as compared to wild type C57Bl/6 macrophages (Figure 1B). These findings indicate that superoxide production requires the presence of cytochrome b558 and the presence of functional FcγR.

Direct correlation between increased nitrite production and a decreased percentage of macrophages infected with *L. major*.

We know $CD4^+$ T cells play a key role during *Leishmania* infection by producing interferon gamma (IFN-γ) which functions to activate infected macrophages to eliminate intracellular pathogens (28). Macrophage IFN-γR binding of INF-γ activates inducible iNOS to produce NO (5). When BMM from IFN-$\gamma R^{-/-}$ and $iNOS^{-/-}$ mice were infected with *L. amazonensis* or *L. major* and co-cultured with TLN cells from *L. major*-infected mice, there was a significant decrease in nitrite production as compared to all other groups (Figure 2A). Analysis of nitrites and percent infected macrophages showed a direct linear correlation between parasite killing and increasing amounts of nitrites from *L. major*-infected BMM (Figure 2B). This finding confirms that production of NO by *L. major*-infected macrophages is necessary to kill the parasite as IFN-$\gamma R^{-/-}$ and $iNOS^{-/-}$ mice are not able to produce nitrite in response to infection. In comparison, when BMM are infected with *L. amazonensis* and co-cultured we do not see a direct linear correlation with NO production (Figure 2B); confirming that NO alone is not sufficient to kill *L. amazonensis*, as previously described (23).

Superoxide is detected in the co-culture system at day 5

Our laboratory has shown that that macrophages require only NO to kill *L. major*, while they require both NO and superoxide to kill *L. amazonensis* (23). In figure 1 we demonstrate that $Fc\gamma R^{-/-}$ and $cytoB^{-/-}$ mice are unable to eliminate *L. amazonensis* infection. Based on this knowledge, we wanted to determine if the production of superoxide occurs temporally to the time killing of *L. amazonensis* within infected macrophages. We previously demonstrated macrophage killing of *L.*

amazonensis in our in vitro system occurs at day 5 following co-culture with either total lymph node cells or purified $CD4^+$ T cells and B cells (22). To detect superoxide in vitro we utilized NBT staining. While a small amount of formazan precipitant was observed early (at 30 minutes), significant production of formazan only occurred at day 5 of co-culture, which corresponds to the time of *L. amazonensis* killing in infected macrophages (Figure 3A and B).

Assembly of NADPH oxidase subunits $gp91^{phox}$ and $p67^{phox}$ occurs early during co-culture and is directly adjacent to the parasite.

The NADPH oxidase complex plays a critical role in phagocytic killing of ingested microorganisms via generation of superoxide. NADPH oxidase subunits will assemble on the membrane of phagosomes immediately during and following phagocytosis (31). Based on the 5 day delay of superoxide production we observe, we wanted to determine the kinetics of NADPH oxidase complex assembly in our system. We harvested *L. amazonensis*-infected BMM 1, 2, 3, 4 and 5 days following co-culture and performed immunofluorescence. We show that co-localization of $gp91^{phox}$, a membrane bound NADPH oxidase subunit, and $p67^{phox}$, a cytosolic subunit, occurs by day 1 of co-culture (Figure 4, Day 1). At all time points measured (days 1-5) co-localization of $gp91^{phox}$ and $p67^{phox}$ was appreciated as observed via confocal microscopy (Figure 4, Day 5 and data not shown) and as measured by Pearson's coefficient of co-localization (Table 1). These data indicate that although NADPH oxidase complexes appear to be assembled by day 1, there is not production of measurable superoxide until day 5 (Figure 3). We additionally wanted

to determine the sub-cellular location of NADPH oxidase gp91^{phox} and p67^{phox} subunit assembly. LAMP1 labeling was used to identify the location of late endosomes, the compartment which would contain the parasite which is also known as the parasitophorous vacuole (PV) (2). We demonstrated that co-localization of gp91^{phox} and p67^{phox} occurred directly adjacent to DAPI-labeled parasite DNA but co-localization was not present on LAMP1 positive PV (Figure 5). This may suggest that NADPH oxidase assembly occurs either on the parasite membrane itself, or on an inner membrane of a multi-lamellar endocytic vesicle, separate from the PV. NADPH oxidase assembly was not occurring at the plasma membrane, but instead at the intracellular compartment containing *L. amazonensis*, which may indicate that superoxide production occurs within established PV's.

Wortmannin inhibits production of superoxide.

Superoxide is generated when the NADPH oxidase complex is assembled and activated (24). In neutrophils production of superoxide is mediated by the p40^{phox} subunit of the NADPH oxidase complex and is dependent upon activation of the PI3K signaling pathway activated by FcγR (31). We previously demonstrated a requirement for FcγR and found that gp91^{phox} and p67^{phox} subunits co-localize without superoxide production at days 1-4 in co-culture. We hypothesized that generation of superoxide may be sensitive to PI3K inhibition. To test this hypothesis we added wortmannin, a PI3K inhibitor, at day 5 of co-culture to macrophages 30 minutes prior to NBT and assessed formazan precipitants. We did not observe formazan precipitants in the presence of wortmannin, indicating superoxide was not

produced (Figure 6A, right and C). In the absence of wortmannin, as previously described, a measurable amount of formazan precipitant was detected (figure 6A, left and C) and macrophage-mediated killing of *L. amazonensis* was observed (figure 6A, right inset). Wortmannin has a short half-life (33) so a time course was performed to determine the incubation time necessary to inhibit parasite killing. When wortmannin was added 3 hours prior to addition of NBT, a small amount of formazan precipitate was measurable (Figure 6B, left and C). This indicates that although wortmannin inhibits superoxide production, it is only for a limited time period and superoxide production appears to be a continual process, so we still observe killing of *L. amazonensis*. To test whether continuous wortmannin treatment would inhibit parasite killing we added the drug twice daily beginning at day 1 of co-culture and continued until day 5. All BMM were then harvested cells at day 5. Cells from the cultures that received twice daily treatments beginning at either day 1 or 2 were not viable, indicating continuous wortmannin treatment for that time period was detrimental to cell viability (data not shown). When wortmannin was added beginning at day 3 and continued twice daily until day 5 we not only did not detect superoxide (Figure 6B, right and C), but we also did not detect any decrease in *L. amazonensis* parasites within infected macrophages (Figure 6B, right inset). These findings indicate that the PI3K pathway is necessary for production of superoxide at day 5 in co-culture and that superoxide production is again, necessary for killing of *L. amazonensis* in our system.

Discussion

Here we propose a novel antibody-mediated mechanism of macrophage activation to kill an intracellular pathogen. Previous work has documented that *L. amazonensis* parasites resist macrophage killing as compared to *L. major* (10, 29). Using an established in vitro model, we have determined that there are two key cell types from the draining lymph node of *L. major*-infected mice required to kill *L. amazonensis* within macrophages; $CD4^+$ T cells and B cells (22) (8). Based on these findings we wanted to determine the means by which these specific cell types could activate infected macrophages to kill the parasite. It is well documented that a polarized T helper 1 (Th1) immune response with activated $CD4^+$ T cells is necessary to activate infected macrophages to kill *L. major* (28). We suggest the role of $CD4^+$ T cells in our system is likely to produce IFN-γ which would bind the IFN-γR to activate iNOS production of NO. When IFN-$\gamma R^{-/-}$ and $iNOS^{-/-}$ macrophages are plated in the in vitro assay we demonstrate that there is neither killing of *L. major* or *L. amazonensis*, indicating this pathway for macrophage activation of NO production is required during both infections (Figure 1A). The role of B cells in our assay was less understood, although it had been determined that antibodies were necessary for parasite killing (22) (8). Superoxide production has been documented primarily during phagocytosis, when assembly of NADPH oxidase is triggered by binding of immunoglobulins to FcγR on the surface of macrophages (12). To determine if this macrophage activation pathway for superoxide is required during *L. amazonensis* infection we utilized BMM from FcγR common chain and cytochrome b558 (cytoB) knockout mice in our assay. When the $Fc\gamma R^{-/-}$ or $cytoB^{-/-}$

BMM are infected with *L. amazonensis* and co-cultured there is not production of superoxide in vitro nor do we observe killing of the parasite (Figure 1A and B). Taken together, these findings indicate that the FcγR common chain on macrophages is required, as is NADPH oxidase, for production of superoxide and thus, killing of intracellular *L. amazonensis* at day 5 of co-culture.

Using the in vitro model (23) we determined there is not a direct correlation between increased nitrite production and killing of *L. amazonensis* in our system, indicating that nitric oxide (NO) alone is not sufficient to kill this parasite (Figure 2B) (23). When superoxide is present in our in vitro system along with NO, we detect killing of *L. amazonensis* in macrophages (23). Here we show that there is detectible superoxide production via NBT on day 5 of co-culture, which corresponds to the day at which we observe parasite killing (Figure 3A and B).

The NADPH oxidase complex is composed of both membrane-bound and cytosolic subunits that assemble for the production of superoxide. Assembly has been well documented to occur in both neutrophils and macrophages at the time of phagocytosis (14, 25). Here we demonstrate that NADPH oxidase co-localization of $gp91^{phox}$ and $p67^{phox}$ occurs at day 1 of co-culture and is present over time until day 5 (Figure 4) (Table 1). Interestingly, we see co-localization of these two subunits directly adjacent to the parasite, but not on the LAMP 1 positive parasitophorous vacuole (PV) (Figure 5). Furthermore, at day 5 we do not find NADPH oxidase complex assembly associated with either identifiable phagocytic cups or small phagosomes that would suggest NADPH activation via FcγR-mediated uptake of antibody-opsonized parasites. Our findings indicate that NADPH oxidase assembly

is either occurring directly on the parasite membrane itself, or on a separate host cell membrane within the PV. During *L. pifanoi* promastigote infection of macrophages the immature form of $gp91^{phox}$ was localized to the PV. The same study showed *L. pifanoi* increased heme degradation which blocked the maturation of $gp91^{phox}$ and prevented NADPH oxidase assembly (26). The PV during *Leishmania* infection is an acidic compartment that acquires molecules such as LAMP1 and 2, rab7 and macrosialin from the endocytic compartment (2, 6). During *L. amazonensis* infection of macrophages the PV is very large and often contains multiple amastigotes which have an attachment site to the PV membrane itself (4). It is possible that *L. amazonensis* amastigotes are encased in a closely-associated host cell membrane that is not part of the larger PV, or a multilamellar body; although this hasn't been documented. Multilamellar bodies are lysosomal organelles which contain multiple layers of cell membranes which can be physiologically normal, such as those that produce surfactant in alveolar type II cells within the lung, or can accumulate during lysosomal storage diseases and autophagy (15). *L. donovani* has been documented to induce autophagic machinery in human bone marrow cells (19), and this coupled with these preliminary findings might suggest *L. amazonensis* infection may be susceptible to superoxide-induced host cell autophagy and multilamellar body formation (11). The other possibility would be that the parasite itself has taken up NADPH oxidase subunits. Uptake of certain host cell components by *Leishmania* species has been documented. MHC class II and H-2M molecules have been documented to undergo internalization and degradation within amastigotes (2). In either case, we show that NADPH oxidase assembly occurs directly on or adjacent

to the amastigote early in infection and this close associate may function to increase the killing ability of superoxide as it would be closely associated with the parasite.

Although NADPH oxidase co-localization of $gp91^{phox}$ and $p67^{phox}$ occurs early in our co-culture system, measurable superoxide production is not appreciated until day 5 (Figure 3). During *L. amazonensis* infection it has been shown that entry of the parasite into host cells occurs via a Rac-1-independent mechanism (20). Rac has a pivotal role during assembly of NADPH oxidase and therefore there may not be NADPH oxidase activation of superoxide during phagocytosis of *L. amazonensis* (20). We did not determine if NADPH oxidase was assembled at the time of phagocytosis, but our findings indicate that there is likely assembly of the complex by day 1 and there is a PI3K-dpendent signal for superoxide production at day 5. In both COS^{phox} cells with transgenes for the separate NADPH oxidase subunits and in human neutrophils it has been shown that the $p40^{phox}$ subunit is essential in stimulating superoxide production (7, 30). A more recent study showed that although NADPH oxidase can be fully assembled, superoxide is not produced until the $p40^{phox}$ subunit stimulates activity of the NADPH complex via a PI3K signal (31). To determine if the PI3K signaling pathway was necessary in our system we treated co-cultured cells with wortmannin, which is a specific inhibitor of PI3K (32). We show that with a 30 minute pre-incubation with wortmannin, superoxide is not detected via NBT, indicating superoxide production is inhibited by the drug (Figure 6A, right and C). We also show that treatment of co-cultured cells for 3 days with twice daily applications of wortmannin not only inhibits superoxide production, but also inhibits killing of *L. amazonensis* (Figure 6B, right and C). We can conclude

that the PI3K pathway is an important signaling mechanism for superoxide production and thus killing of the parasite.

We report here that during an established infection with *L. amazonensis* amastigotes, macrophages can become activated to produce superoxide via FcγR, NADPH oxidase and PI3K dependent mechanism and thus kill the parasite in combination with NO. FcγR-mediated intracellular killing has been documented during *Staphlococcus aureus* infection in human monocytes (36) and the signaling pathway for activation of killing was protein kinase C (PKC), which can be activated by PI3K (34). These studies observed killing of bacteria during a short time period immediately following phagocytosis (34-36), while in our case we see activation of this pathway late in infection. We propose antibodies in our system are produced by B cells following stimulation with *L. major* antigen and adequate amounts of these antibodies are perhaps not produced until day 5 in co-culture. These antibodies then engage FcγR in a manner that can activate pre-assembled NADPH oxidase complexes associated with intracellular parasites. We suggest this pathway of macrophage activation during infection with intracellular pathogens be termed antibody-enhanced intracellular killing. This pathway is another example of the role of antibodies during infection and describes a role for extracellular antibodies in macrophage activation and killing of intracellular organisms within an intracellular compartment.

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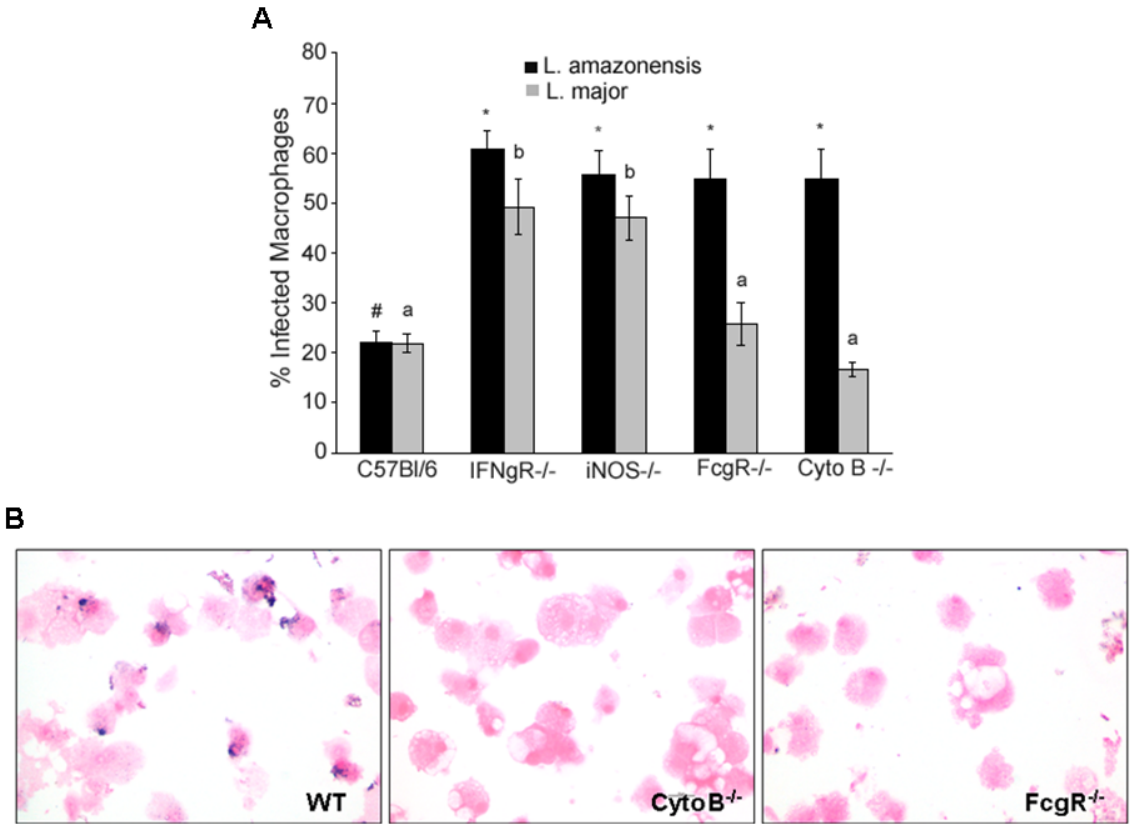

Figure 1. ***L. amazonensis*-infected macrophages require IFN-γR, iNOS, FcγR, and CytoB for parasite killing. A.** BMM infected with *L. amazonensis* or *L. major* and co-cultured with total lymph node cells from *L. major*-infected mice. Parasite number was manually determined via light microscopy. For *L. amazonensis* infection different symbols represent a statistically significant difference ($p < 0.001$) and for *L. major* infection different letters indicate a statistically significant difference ($p < 0.05$). **B.** Cultures were incubated with NBT for 90 minutes at day 5. Basophilic cytoplasmic precipitate (formazan) is indicative of superoxide production within infected cells as measured by nitro blue tetrazolamide (NBT). Cells counterstained with eosin and viewed using light microscopy at 40x magnification. Results are representative from three separate experiments.

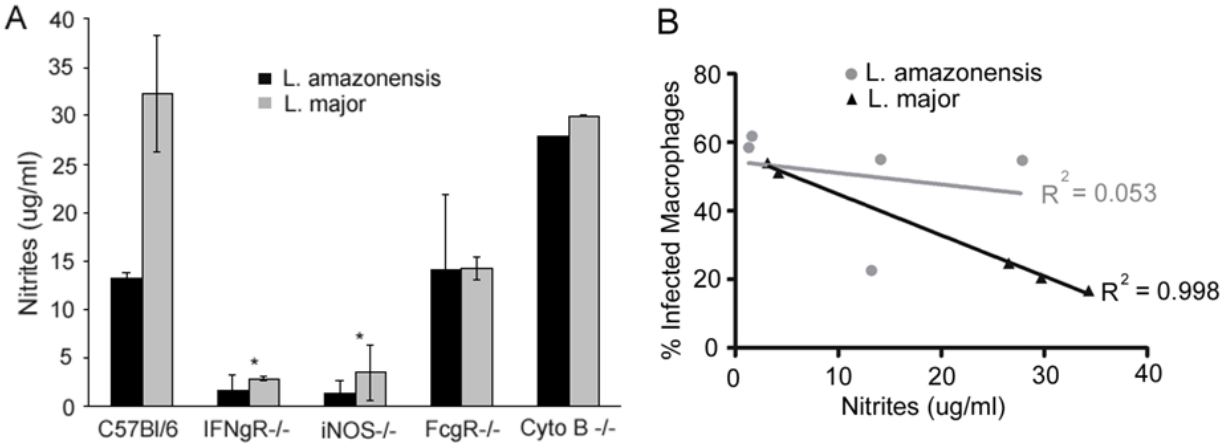

Figure 2. **Decreased nitrite production from macrophages lacking IFN-γR and iNOS and direct correlation between increased nitrite production and *L. major* killing by infected macrophages. A.** Nitrite production as measured by greiss assay. Macrophages were infected with *L. amazonensis* or *L.major* and co-cultured with purified $CD4^+$ T cells and $CD19^+$ B cells from mice infected with *L. major*. * represent statistically significant differences ($p < 0.01$). Results are from three separate experiments ± SEM. **B.** Correlation of nitrite production and the percentage of infected macrophages. There is a linear correlation (R^2=0.998) between the percentage of infected macrophages and nitrite production during *L. major* infection but not during *L. amazonensis* infection (R^2=0.0530), indicating nitric oxide isn't sufficient for killing *L. amazonensis*. Results are from two to three separate experiments.

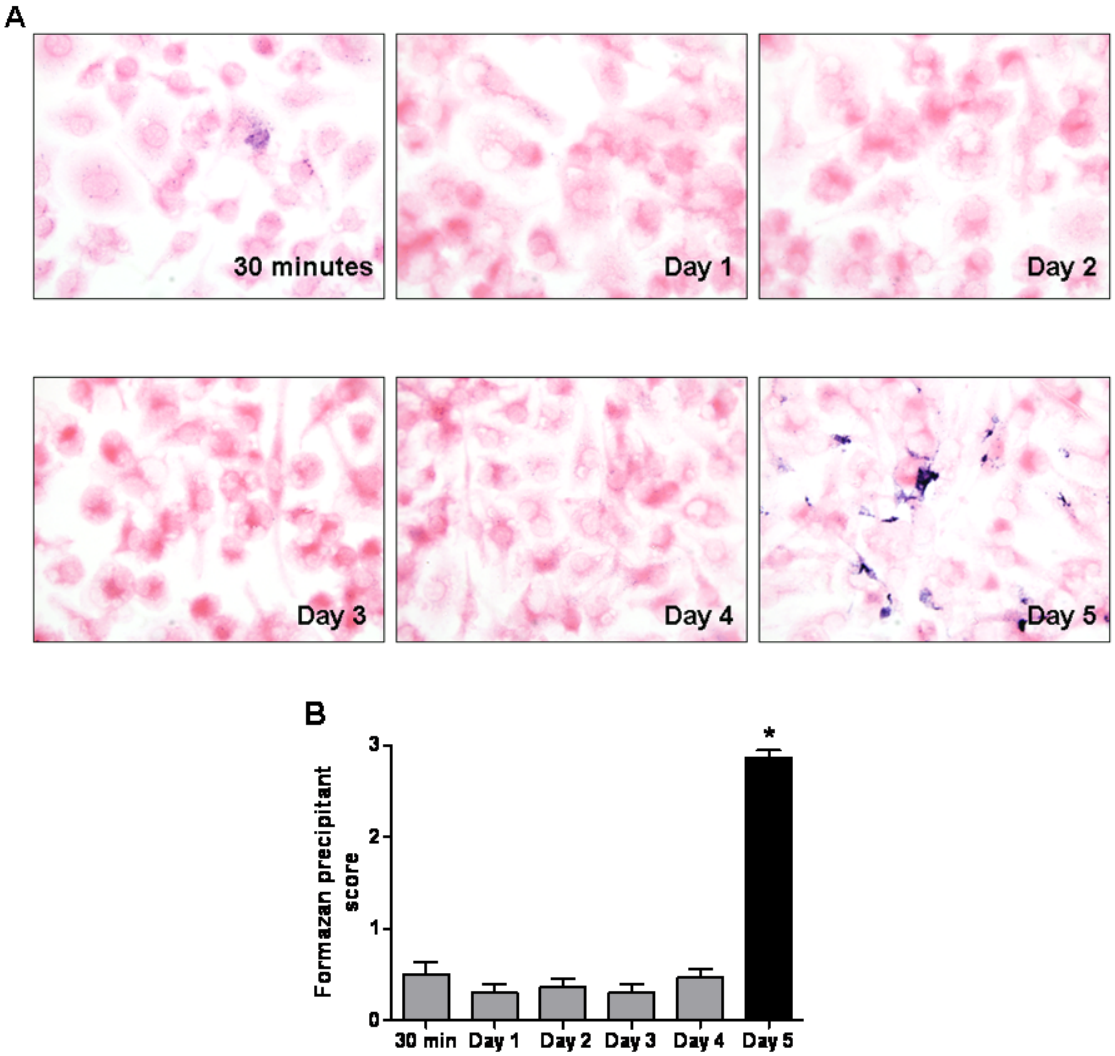

Figure 3. **Lack of significant superoxide production from *L. amazonensis* - infected bone marrow-derived macrophages until day 5.** BMM were infected with *L. amazonensis* amastigotes for 24 hours, washed to remove extracellular parasites and co-cultured with TLN cells from a C3H mouse infected for 4 weeks with *L. major* along with freeze-thawed *L. major* promastigote antigen and cultured at 37°C, 5% CO_2. Cultures were incubated with NBT for 90 minutes at days indicated. Basophilic cytoplasmic precipitate (formazan) is indicative of superoxide production within infected cells as measured by NBT. **A**. Cells counterstained with eosin and viewed using light microscopy at 40x magnification. **B**. Score based on the number of cells with basophilic formazan precipitants. Values expressed as mean value ± SEM. *P < 0.05. Results are from three separate experiments.

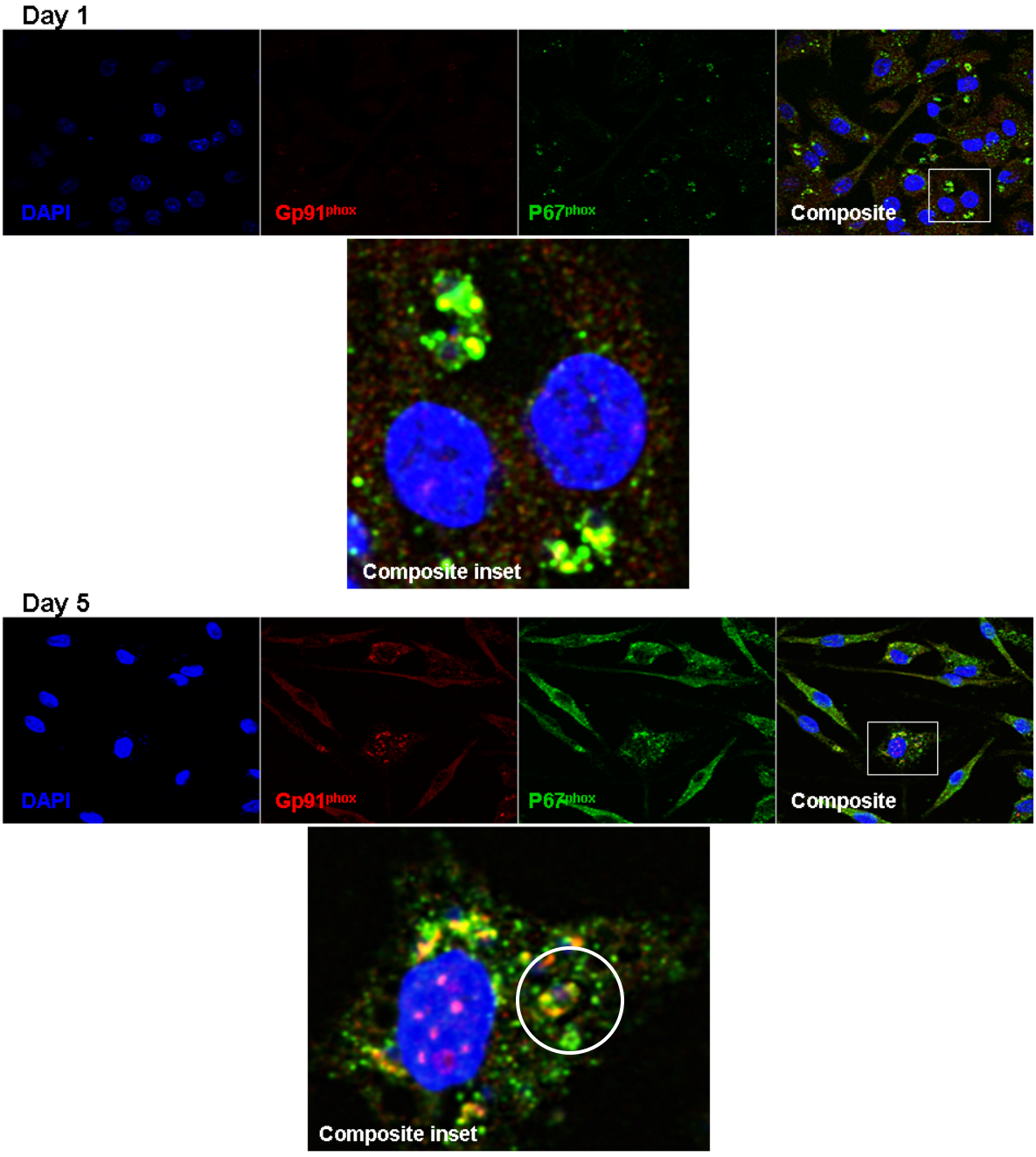

Figure 4. Co-localization of gp91^{phox} and p67^{phox} at days 1 and 5 of co-culture. BMM infected with *L. amazonensis* and co-cultured with TLN cells from *L. major*-infected mice. Coverslips were recovered at the indicated time points, fixed and labeled with gp91^{phox} (red), p67^{phox} (green) and DAPI (blue). Sequential scanning confocal microscopy (x60, oil) analysis of gp91^{phox} and p67^{phox} at 1 and 5 days in co-culture. Co-localization is represented in yellow (composite inset). White circle marks parasite presence (blue) surrounded by co-localized subunits (yellow). Data is representative of three separate experiments.

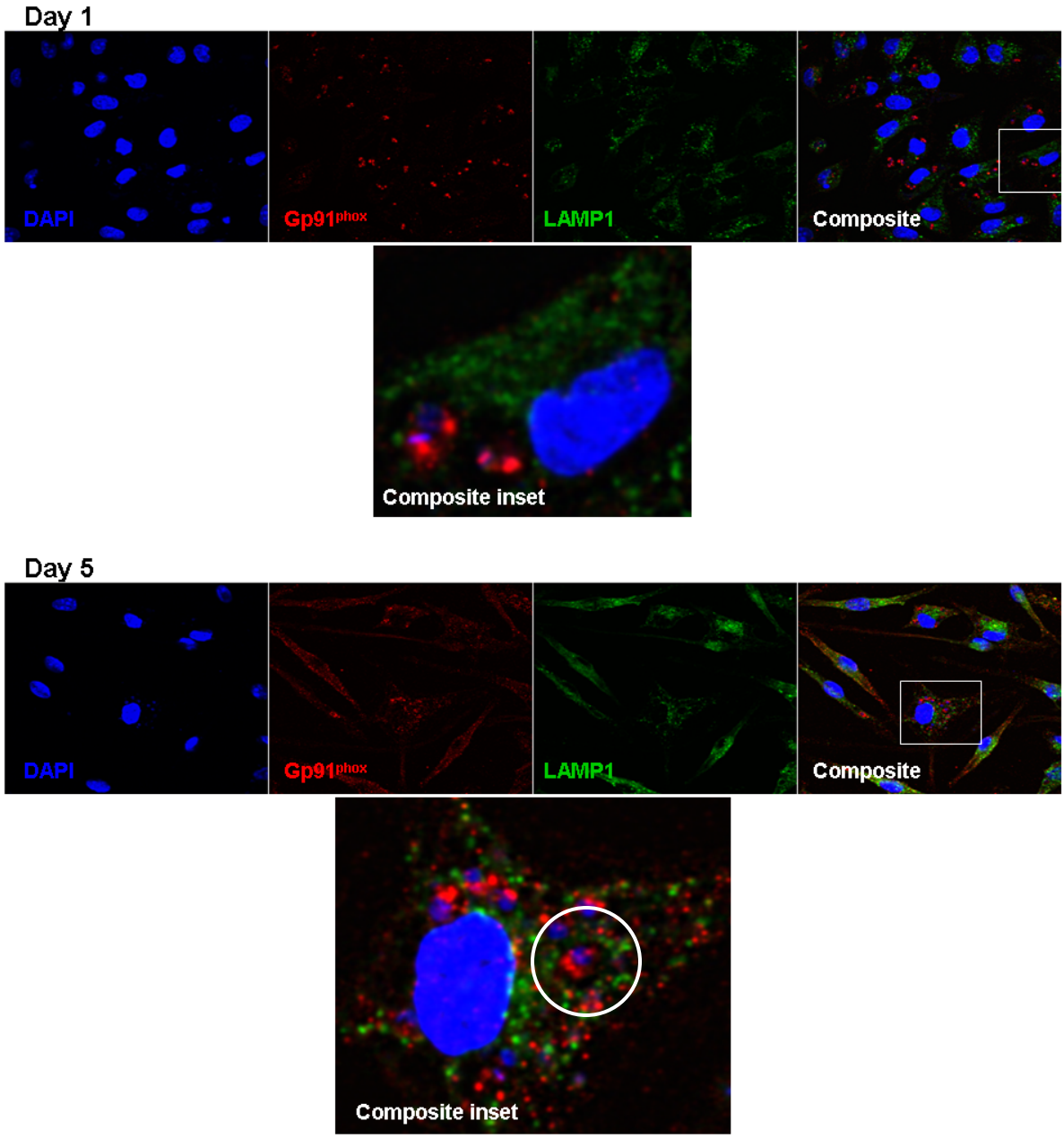

Figure 5. No co-localization of gp91^{phox} and LAMP1 at days 1 and 5 of co-culture. BMM infected with *L. amazonensis* and co-cultured with TLN cells from *L. major*-infected mice. Coverslips were recovered at the indicated time points, fixed and labeled with gp91^{phox} (red), LAMP1 (green) and DAPI (blue). Sequential scanning confocal microscopy (x60, oil) analysis of gp91^{phox} and LAMP1 at 1 and 5 days in co-culture. White circle marks parasite presence (blue) surrounded immediately by gp91^{phox} (red) without LAMP1 (green). Data is representative of two separate experiments.

Day 1	Day 2	Day 3	Day 4	Day 5
0.78	0.65	0.80	0.76	0.82

Table 1. Summary of Pearson's co-localization coefficients . Calculated by Olympus Fluoview version 2.1b software analysis of $gp91^{phox}$ and $p67^{phox}$ co-localization. Score of 1 indicates perfect co-localization, 0 indicates random co-localization is occurring and -1 indicates exclusion. Results are of a single field of view from one experiment and the trend was confirmed in 3 separate experiments.

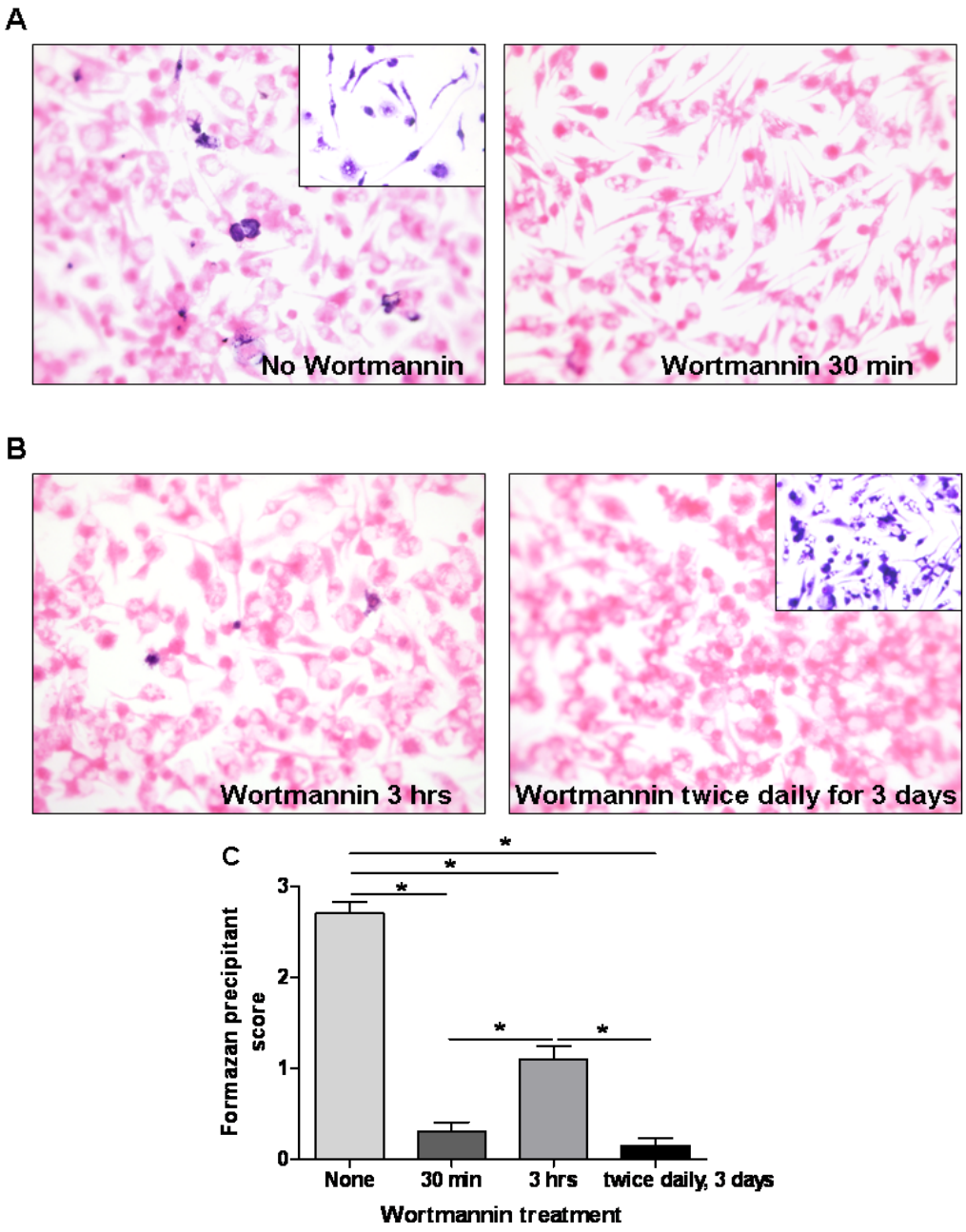

Figure 6. Wortmannin inhibits production of superoxide and killing of *L. amazonensis* at day 5. A. Cultures were incubated with NBT for 90 minutes at day 5, with or without a 30 minute pre-incubation of 100nM wortmannin. Inset: diff-quick stained photomicrograph at day 5. **B.** Cultures were incubated with NBT for 90 minutes at day 5, with a pre-incubation, as indicated, of 100nM wortmannin. Cells counterstained with eosin. Inset: diff-quick stained photomicrograph at day 5 with wortmannin treatment twice daily for 3 days. **C.** Score based on the number of cells with basophilic formazan precipitants. Values expressed as mean value ± SEM. *P < 0.05. Results are from two separate experiments.

Chapter 5

GENERAL CONCLUSIONS

Summary

The work presented in this dissertation demonstrates that B cells play a necessary role in an effective Th1-mediated immune response towards *Leishmania amazonensis*. We studied the host immune response of two mouse strains, C3HeB/FeJ (C3H) and C57Bl/6 (B6), to a co-infection with *L. major* and *L. amazonensis*. Our initial findings indicated a differential immune response between these two strains; C3H mice were able to control infection while B6 mice developed chronic, non-healing lesions. Using an in vitro co-culture model, we demonstrate that the inability of B6 mice to heal a co-infection with *L. major* and *L. amazonensis* correlates with a defect in the B cell response, rather than in the $CD4^+$ T cell response. Overall, our findings indicate that B cells and their antibodies are necessary, but not sufficient, for killing *L. amazonensis* and their antibodies are just one of several critical immune components required for killing.

Analysis of the B cell population of both C3H and B6 mice during co-infection indicates there is a difference in the germinal center B cell response during co-infection with both *L. major* and *L. amazonensis*. We demonstrate that at 2 and 5 weeks post-infection B6 mice have fewer germinal center B cells, fewer germinal center B cells that have undergone isotype switching, fewer memory B cells and fewer antigen-specific IgG2c-producing cells in the draining lymph node, as compared to C3H during co-infection. We also show that IL-21 production in both mouse strains is similar at 2 weeks, indicating the differences we see between these

mouse strains is perhaps due to an intrinsic B cell defect, rather than a defect in the production of IL-21 within germinal centers. To heal an *L. amazonensis* infection, mice must have activated B cells. The deficit within B6 mice that prevents healing of the co-infection may be partly due to an intrinsic B cell defect within the germinal center.

Utilizing our in vitro co-culture system two key findings have been discovered; (1) there are 2 specific cell types from the draining lymph node of *L. major*-infected mice to resolve of *L. amazonensis* infection; $CD4^+$ T cells and B cells (7) and (2) macrophages require both nitric oxide (NO) and superoxide to kill intracellular *L. amazonensis* (8). To determine the mechanism of these two cell types in activation of infected macrophages we first infected macrophages from knockout mice (IFN-$\gamma^{-/-}$, $iNOS^{-/-}$, $Fc\gamma R^{-/-}$, $CytoB^{-/-}$) with *L. amazonensis* or *L. major*. We determined that interferon gamma (IFN-γ) receptor, Fc gamma receptor (FcγR) common chain, inducible nitric oxide synthase (iNOS) and cytochrome B subunit of NADPH oxidase (CytoB) are all necessary for macrophage activation and killing of *L. amazonensis*. NADPH oxidase assembly of $gp91^{phox}$ and $p67^{phox}$ occurs early in vitro, by day 1, and assembly occurred directly adjacent to the amastigote form of the parasite. Although NADPH oxidase was assembled early, measurable superoxide production was only detectable at day 5 in vitro, indicating there was a signal or signals for superoxide production other than complex assembly. Using wortmannin, a phosphoinositide 3-kinase (PI3K) inhibitor, we demonstrate inhibition of superoxide production at day 5. This indicates that PI3K may be required for superoxide production at this late stage of infection. There appear to be two main mechanisms

by which infected macrophages are activated to kill *L. amazonensis*. The first requires *L. major*-specific $CD4^+$T cells to produce IFN-γ that binds IFN-γ receptors to induce NO production via iNOS. The second pathway requires *L. major*-specific B cell production of antibodies that may bind Fcγ receptors and likely trigger PI3K, triggering activation of assembled NADPH oxidase to produce superoxide. This second mechanism is a novel pathway for macrophage activation to kill an intracellular pathogen in which extracellular, antibodies appear to signal the infected cell to produce intracellular superoxide for killing.

Discussion

A deficient germinal center B cell response by C57Bl/6 mice correlates with loss of macrophage-mediated killing of *L. amazonensis*.

Infection of C3H mice with *L. amazonensis* leads to chronic disease with large non-resolving cutaneous lesions and high parasite loads (4) while the same mouse infected with *L. major* stimulates a healing cell-mediated immune response (10). Our laboratory and others have shown that Th1 immunity associated with *L. major* infection provided significant protection against subsequent *L. amazonensis* infection (2, 12, 13). Similar to the cross-protection observed in C3H mice, B6 mice first infected with *L. major* and subsequently challenged with *L. amazonensis* either have small lesions (unpublished observations) or healed lesions, but interestingly, B6 mice do not heal a simultaneous infection with both *L. major* and *L. amazonensis* (1, 2). Our data extends the knowledge concerning why B6 mice do not heal a simultaneous co-infection by first defining that B cells from infected B6 mice are

ineffective in promoting parasite killing compared to B cells from infected C3H mice (1). We demonstrate that the B cell defect during co-infection corresponds to a poor germinal center response within the draining lymph node as measured by the number of germinal center B cells, isotype-switched B cells within the germinal center, memory B cells, and antigen-specific antibody-producing cells. Altogether, these findings indicate that B cells and their antibodies play a key role during *Leishmania amazonensis* infection and the B cell response, specifically the germinal center response differs between mouse strains.

Antibody-Enhanced Intracellular Killing

During an established infection with *L. amazonensis* amastigotes, macrophages can become activated to produce superoxide via an FcγR, NADPH oxidase and PI3K-dependent mechanism to kill the parasite in combination with nitric oxide (NO). The pathway by which NO is generated within infected macrophages is well established. Macrophages are classically activated by IFN-γ produced by $CD4^+$T cells, along with exposure to microbes or microbial products, and are identified via production of nitric oxide (NO) (5, 6). Here we demonstrate the role of $CD4^+$ T cells is to produce IFN-γ which binds the IFN-γR to activate iNOS production of NO (Figure 1). When IFN-$\gamma R^{-/-}$ and $iNOS^{-/-}$ macrophages are utilized in our in vitro co-culture assay we show that there is neither killing of *L. major* nor *L. amazonensis*, confirming the importance of this pathway in activation of the *Leishmania*-macrophage and subsequent production of NO to remove intracellular parasites.

The novel mechanism of *Leishmania*-infected macrophage superoxide production was determined as a part of this dissertation. Superoxide was primarily thought to be generated immediately during phagocytosis when NADPH oxidase complexes were assembled upon the phagosomal cup and membrane (9). Phagocytosis can be initiated via FcγR-ligation of opsonized particles as previously established (11). We had already determined that B cells were a necessary part of a productive immune response by C3H mice against *L. amazonensis*. We thus wanted to further determine the mechanism by which B cells induce superoxide production in activated macrophages. We determined that FcγR common chain and NADPH oxidase complex were required to kill *L. amazonensis* at day 5 in vitro. Interestingly, while NADPH oxidase complex formation occurred by day 1 as determined by co-localization of $gp91^{phox}$ and $p67^{phox}$, measurable superoxide production was not observed until day 5 of co-culture which is much later than the initial phagocytic event. Based on this finding and the knowledge that NADPH oxidase was assembled at early time points (day 1) we showed via wortmannin inhibition that the PI3K signaling pathway is likely required for superoxide production at this late time point. A recent study described that, although assembly of the NADPH oxidase complex can occur within neutrophils, production of superoxide required the $p40^{phox}$ subunit of the complex and PI3K signaling activation (11). We would propose that superoxide production via this pathway is also occurring in *L. amazonensis*-infected macrophages at day 5 of in vitro co-culture. Taken together, all of these findings suggest there is an extracellular signal, most likely triggered by binding of antibodies to FcγR that results in wortmannin-sensitive superoxide

production by NADPH oxidase complexes. We know B cells are necessary in our in vitro co-culture system for intracellular parasite killing, as is *L. major* antigen, and therefore propose antigen-antibody complexes produced in the system over time bind to stimulatory FcγR on the macrophage surface to trigger intracellular killing of *L. amazonensis* via PI3K and pre-assembled NADPH oxidase-dependent signals (Figure 1). We hypothesize these immune complexes are small and therefore are internalized with FcγR endocytosis as opposed to phagocytosis. This would be consistent with the fact that we do not see $gp91^{phox}$ and $p67^{phox}$ co-localization at the plasma membrane surface, which would be expected if phagocytosis of these complexes was occurring. FcγR-mediated endocytosis is not dependent on the cytoskeleton, so we should be able to block FcγR-mediated phagocytosis by cytochalasin-D, an inhibitor of actin polymerization, and still observe macrophage activation and parasite killing (3). FcγR-mediated intracellular killing has been documented during *Staphylococcus aureus* infection in human monocytes (14), but not at such a late time point as observed in our system (day 5). This role for antibodies in killing of *L. amazonensis* could likely be applied to any intracellular pathogen associated with pre-assembled NADPH oxidase complexes and represents a novel mechanism for macrophage activation.

Recommendations for future studies

One of the future goals of this research is to understand why B6 mice do not heal a co-infection with *L. major* and *L. amazonensis* as compared to C3H mice. Additional experiments are necessary to determine the mechanism behind the

germinal center defect during early infection of B6 mice. Although we have evidence that there is adequate IL-21 production in B6 mice, it would be recommended that the Tfh cell population be analyzed in both mouse strains to determine if there is a difference in the number of Tfh cells present within germinal centers. It would also be necessary to determine if there is a difference in surface expression of the IL-21 receptor on B cells because although there may be adequate production of IL-21 by Tfh cells, B6 B cells may have a decreased expression of the receptor and therefore decreased IL-21 stimulation. Continued exploration of the B cell compartment of B6 mice is also warranted, as it appears that it may not be simply their production of IgG2c as compared to IgG2a by C3H mice that leads to the differential healing phenotype, but instead a difference in the germinal center response. Additionally, in vivo experiments would be suggested to determine if it is possible to overcome the B cell defect during co-infection of B6 mice. An F1 generation of B6 x C3H mice could be generated for cell transfer experiments. These mice could be irradiated and then reconstituted with $CD4^{+}$T cells from either co-infected C3H or B6, as well as B cells from co-infected C3H mice to determine if healing would occur. A similar experiment could be performed with the transfer of B cells from co-infected B6 mice and we would hypothesize these mice would develop non-healing chronic lesions.

A second goal for the research presented in this dissertation is to better characterize the mechanism of antibody-enhanced intracellular killing. First and foremost, we could confirm the PI3K signaling pathway is activated by day 5 by performing western blot analysis of phosphorylated AKT (pAKT) over time. We would hypothesize that pAKT would not be detected early but would be present at

day 5 of co-culture when we observe measurable superoxide production. Additional experiments are also necessary to better define the pathway by which extracellular antibodies or antigen-antibody complexes activate PI3K and to determine if PI3K is activating the $p40^{phox}$ subunit of the NADPH oxidase complex to produce superoxide. Immunofluorescence could be used to track antigen-antibody complexes from the extracellular membrane, after which they may be taken up via FcγR-mediated phagocytosis. If so, we would like to assess trafficking of these complexes to determine if they are targeted to the parasitophorous vacuole. FcγR-mediated phagocytosis of these complexes may not occur, as we do not see NADPH oxidase assembly at the membrane of macrophages at day 5, indicating phagocytosis is likely not occurring. If this is the case, we might hypothesize antibody binding of these receptors simply triggers an intracellular signal that activates PI3K. We would propose this intracellular signal would be activated by FcγR-mediated endocytosis and to test this we could block FcγR-mediated phagocytosis by cytochalasin, an inhibitor of actin polymerization. We would expect to still observe killing of *L. amazonensis*. Experiments would also be warranted to determine if the other components of the NADPH oxidase complex are assembled early during infection, including the $p40^{phox}$ subunit, as this is the subunit required for superoxide production via PI3K signaling. We would also like to better characterize the cell membranes upon which $gp91^{phox}$ and $p67^{phox}$ are assembled. We could use immunofluoresence to determine if these membrane-bound compartments are early endosomes ($Rab5^+$), late endosomes ($Rab7^+$) or recycling endosomes ($Rab11^+$). Finally, it would be interesting to determine if other intracellular pathogens that

cause chronic disease, such as but not limited to *Brucella*, *Mycobacterium* and/or *Rhodococcus* can be killed via antibody-enhance intracellular killing. If in fact this is true, we may be better able to target treatment strategies that either activate infected cells to themselves kill the pathogen or target drugs to the intracellular compartment containing the pathogen; thus aiding in the development of treatments and/or cures for chronic disease caused by intracellular organisms.

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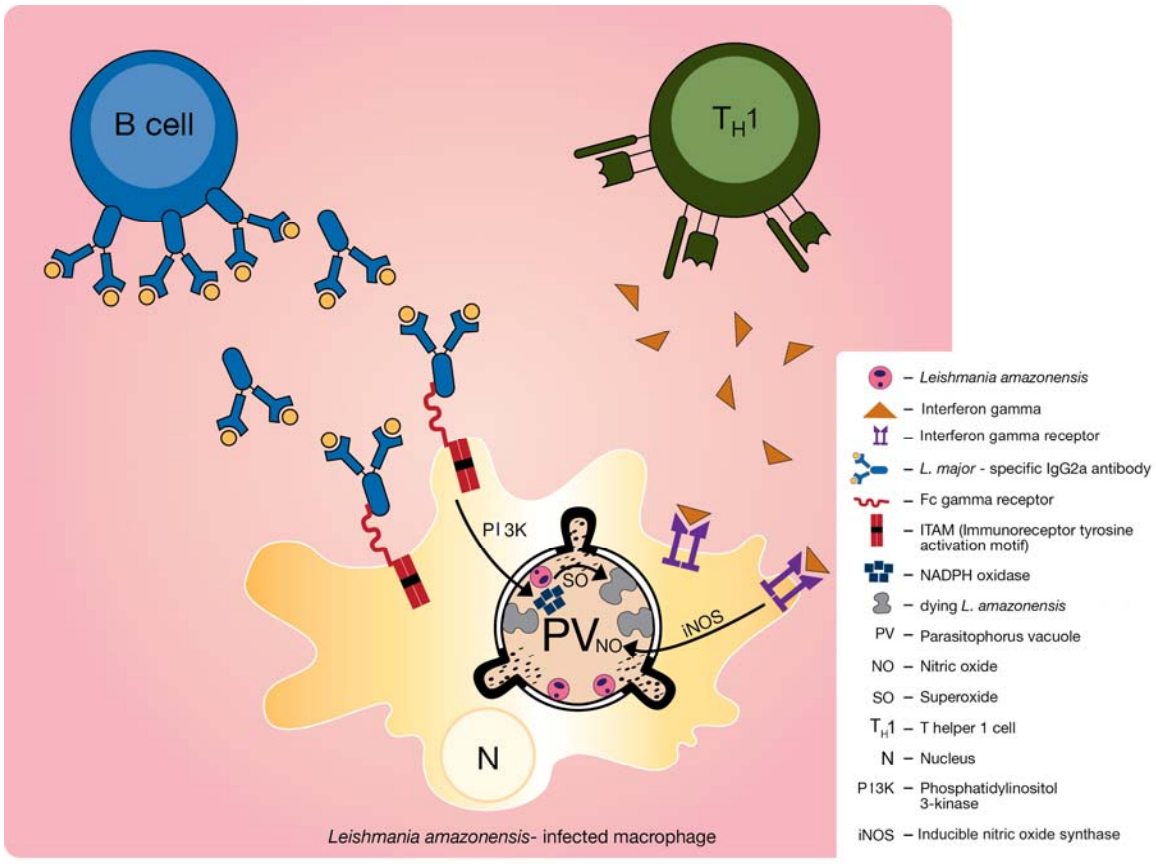

Figure 1. Proposed model for activation of *L. amazonensis*-infected macrophages by *L-major* derived CD4$^+$ T cells and B cells. Bone marrow-derived macrophages are infected with *L. amazonensis* amastigotes and then co-cultured with CD4$^+$T cells and B cells derived from an *L. major*-infected mouse. CD4$^+$T cells produce IFN-γ that binds IFN-γ receptors and activate iNOS production of nitric oxide within the parasitophorous vacuole. B cells produce antibodies that bind stimulatory Fcγ receptors on the macrophage that via PI3K signaling, activate assembled NADPH oxidase to produce superoxide in the parasitophorous vacuole. The combination of nitric oxide and superoxide is lethal to *L. amazonensis*.

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